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*PROTEIN TURNOVER IN MICRO-ORGANISMS**

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Communicated March 3, 1958

The existence of the dynamic state of tissue constituents of living organisms is one of the few general patterns of biological systems which have emerged during the past few decades. It would be surprising, therefore, if the dynamic state which had been observed in the tissues of many species of Metazoa should be absent in micro-organisms. Such a tentative conclusion was suggested from studies of the synthesis and disappearance of the inducible enzyme β-D-galactosidase by Hogness, Cohn, and Monod.[1]

These authors found, in an investigation on the synthesis of the inducible enzyme β-galactosidase in *Escherichia coli,* that its synthesis did not utilize amino acids resulting from the breakdown of cellular proteins. On the basis of these experiments they suggested that the dynamic state described by Schoenheimer, Ratner, and Rittenberg[2] in mammals does not exist but that these investigators were merely observing the total breakdown and dissolution of cells with the concomitant synthesis of completely new cells. The turnover rate determined by Schoenheimer, Ratner, and Rittenberg was ascribed to the rate of synthesis of new cells to replace those worn out. In essence, Hogness, Cohn, and Monod have revived the wear-and-tear theory. This work has been reviewed by Kamin and Handler[3] and by Tarver.[4]

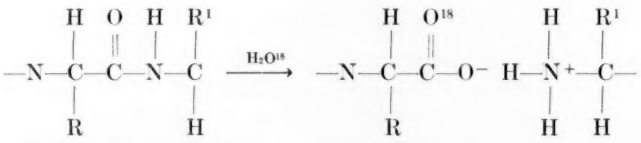
The theory of the dynamic state does not rest solely on the regeneration of proteins. Turnover has been observed with the fats, the carbohydrates, and the proteins. It has been observed in vivo and in vitro, and it applies to the amino acids, the fatty acids, and the monosaccharides, as well as to the most complex constituents of the cell. The rate is rapid in some tissues and compounds as, for example, rat liver and glutathione, and slow in others, such as human muscle.[5, 6]

It must be remembered that the theory of the dynamic state was developed to describe systems whose mass was either stationary or slowly varying. Slow growth, for example, could be explained by a decreased degradative reaction with an unaffected synthetic rate. Direct application of this theory to a bacterial system in logarithmic growth is not immediately justified. In the experiments of Hogness *et al.*, the bacterial mass was doubling each hour. It seems reasonable to deduce that there is a profound difference in the synthetic mechanisms, or their controls, between the non-growing *E. coli* or mammal and the cell in logarithmic growth. The results obtained on cells in logarithmic growth might not apply to cells in the non-growing state, let alone to the mammal. It seemed of interest to study this question

in the non-growing micro-organism preparatory to investigating it in cells in logarithmic growth.

Previous studies by other investigators have attacked this question by using labeled amino acids to label cell proteins and then determining by more or less indirect methods whether labeled amino acids are liberated from the labeled protein.[1, 7, 8] We have studied this question using a direct procedure.

We have determined the rate of protein turnover in *E. coli* K_{12} W · 6 during methionine starvation, using both O^{18} and deuterium-labeled water in the medium. The incorporation of an amino acid into a pre-existing protein requires the hydrolysis of at least one peptide link and its re-formation. The hydrolysis of a peptide link in an environment of H_2O^{18} will result in the introduction of O^{18} into the carboxyl group formed:



Because of resonance, both oxygen atoms in the carboxylate ion would be equally labeled and contain but half the isotope concentration of the medium. Re-formation of the peptide bond would remove one of the two equivalent oxygen atoms and yield a peptide bond containing half the O^{18} concentration of the water. Repetition of this process would result in the O^{18} concentration of the oxygen of the peptide bond eventually becoming equal to that of the medium.

There is no known mechanism by which deuterium from the water of the medium can be introduced into stable positions in the protein by an enzyme-catalyzed reaction. However, free amino acids, under the action of transaminase, rapidly incorporate deuterium in the α-position.[9, 10] The reincorporation of the amino acid into the protein will result in the appearance of deuterium in the protein. If the rate of the incorporation of deuterium into the α-position of the amino acid is very much more rapid than the rate of hydrolysis and re-formation of the peptide bond, then the appearance of deuterium in the protein will be a measure of the turnover of the protein. The rate at which deuterium will appear in the protein will thus offer a minimum value for the rate of protein turnover.

In these experiments no substance was used which would impose a novel condition on the micro-organisms which had been previously adapted to a specific, well-defined milieu. Nor were there diffusion barriers or problems connected with active transport to complicate the interpretation.

In order to simplify the conditions, it was desirable that there be no protein synthesis; of course, the organisms must remain viable during the course of the study. The auxotroph *E. coli* K_{12} W · 6 is known to fulfil these requirements. It is an extraordinarily stable auxotroph. Twelve years after its isolation[11] it shows no detectable tendency to back-mutation to prototrophy. Not only is the organism unable to increase the amount of its protein during the period of starvation of its essential nutrient, methionine, but even after the restoration of the amino acid there is a lag of about 90 minutes before protein synthesis is resumed.[12] The viability of the organism during several hours of starvation has been established.[12]

Experimental.—*E. coli* K_{12} W · 6 were grown on a chemically defined medium[13] and were harvested during the logarithmic growth phase. They were then incubated for 100 minutes in a similar medium lacking methionine to exhaust the amino acid pool of this amino acid and to establish an internal condition in which no net protein synthesis is possible. The organisms were then transferred to a fresh methionine-free medium containing a known concentration of D_2O^{18}—13 per cent D; 1.4 per cent O^{18}—and incubated aerobically. At hourly intervals bacteria were isolated by centrifugation at 5° C. and were immediately extracted with 5 per cent trichloroacetic acid. For the isolation of the protein fraction the procedure described by Roberts *et al.*[14] was followed, with the exception that the extraction with hot trichloroacetic acid was repeated. This precaution was essential to insure the removal of the final traces of nucleic acids. When $P^{32}O_4^{\equiv}$ was incorporated into the culture medium of the organisms, some residual radioactivity was still measurable after a single extraction with hot trichloroacetic acid. However, after the second extraction, this became negligible, indicating less than 0.2 per cent residual nucleic acids. The protein was dried at 100° *in vacuo* for 2 hours. Four to 6 mg. of the dried product were analyzed for its O^{18} content by the method of Rittenberg and Ponticorvo.[15] The proteins contained no detectable O^{18} in experiments in which normal cells were transferred to 1.4 atom per cent excess O^{18} water and were immediately killed and analyzed as above. This demonstrates that O^{18} found in proteins was not the result of adsorbed water or water of hydration.

Data are given in Table 1 to demonstrate that the cells were still viable after 2 hours. The presence of dinitrophenol had no permanent toxic effect. To demonstrate that these cells, under our conditions, are incapable of *de novo* protein synthesis, an aliquot of the bacteria without dinitrophenol was incubated for 2 hours in a medium containing lactose but no glucose or methionine. The concentration of β-D-galactosidase in these cells was assayed by determining the amount of *o*-nitrophenol produced per hour per 10^8 cells from *o*-nitrophenyl-β-D-galactoside.[16] The value, 0.3 μg., was not larger than values found in these organisms not exposed to the inducer.

TABLE 1

THE VIABILITY OF *E. coli* K_{12} W · 6 DURING STARVATION OF METHIONINE

Adjuvant	0	2 Hr.
...	9.0×10^4	8.6×10^4
Dinitrophenol 10^{-3} *M*	8.6×10^4	8.6×10^4

Discussion.—While it is reasonable to assume from general theoretical considerations that the incorporation in stable positions of both O^{18} and D into the proteins is the result of an enzymatic process in an intact cell, we are able to offer direct experimental evidence for this. In the presence of 10^{-3} *M* dinitrophenol, cells incubated in D_2O^{18} incorporate only traces of isotope (see Fig. 1). This concentration is known to uncouple oxidative phosphorylation. The inhibition is not unexpected, since the mechanism for the isotope incorporation involves the formation of peptide bonds, a process known to require chemical energy.

In the absence of dinitrophenol, both O^{18} and D are incorporated into proteins. To calculate the rate of turnover of the proteins, it is necessary to know the rate of change of the O^{18} concentration of the protein and the maximum value which could

be attained. Repeated opening and closing of the peptide bond must eventually equalize its O^{18} concentration with that of the water. However, not all of the protein oxygen is present in the peptide bond; some oxygen is found as hydroxyl groups or as the free carboxyl groups of aspartic and glutamic acid. The repeated opening and reclosing of the peptide bonds will have no effect upon the isotope concentration of the non-peptide bond oxygen. The exact composition of the mixture of bacterial proteins is not known. However, for insulin and β-corticotrophin—two proteins whose exact structure is known—the peptide oxygen accounts for 64 and 67 per cent of the total oxygen, respectively. For the purposes of this paper, we shall assume that two-thirds of the total oxygen of the bacterial proteins which we analyzed is peptide oxygen. Similarly, we estimate, using insulin and β-corticotrophin as models, that in the average protein the α-hydrogen accounts for 12.5 per cent of all the hydrogen atoms.

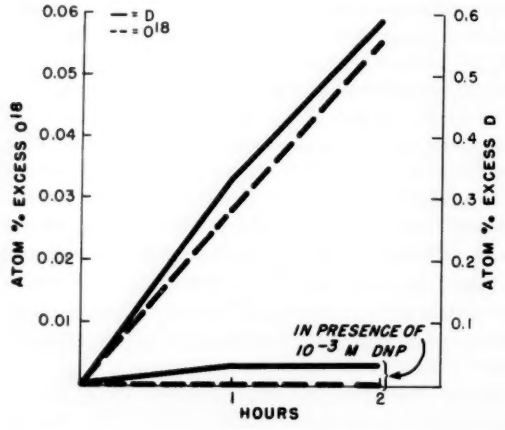


FIG. 1

The data of Figure 1 show that the uptake and therefore the turnover rate are linear during the 2 hours of methionine starvation. Using the estimate that two-thirds of the total protein oxygen is peptide-bond oxygen, the maximum O^{18} concentration that the protein of the non-growing cell could reach would be $2/3 \times 1.4 = 0.93$ atom per cent excess. The rate of increase of O^{18} in the protein corresponds to a turnover of 3 per cent per hour (Fig. 1). The opening of a peptide bond leads to the introduction of one oxygen atom from the medium, but the formation of a peptide bond can take place with either one of the two identical atoms of the carboxylate ion. The result of the opening and reclosing of a peptide bond will be, on the average, that half of the peptide bonds will contain an oxygen atom derived from the medium. Because only half of an O^{18} atom is introduced in the peptide bond as a result of the opening and reclosing of a peptide link, our estimate of the rate of the reaction should be doubled. The turnover will thus amount to 6 per cent per hour. If, however, the carboxyl group of the amino acid while free can undergo exchange under the action of the proteolytic enzyme,[17] then the turnover rate will be less. At minimum, the rate is 3 per cent per hour; at maximum, 6 per cent per hour. Even accepting the lower value, 3 per cent per hour, the rate of protein turnover is considerable.

The rate of incorporation of deuterium into the protein is about 0.33 atom per cent excess per hour. It is difficult from our present data to fix the maximum isotope concentration to be expected in the protein while in the free state. If deuterium is present only in the α-position of the amino acid residues, then the maximum value could be about one-eighth that of the medium or $13/8 = 1.62$ atom per cent

excess. This corresponds to a turnover of $0.3/1.62 \times 100$ or 20 per cent per hour. If deuterium is present in other positions of the amino acid residue as well as the α-position, then the calculated turnover rate will be lower. A minimum value can be obtained from the following data. These bacteria, when grown up from a small inoculum in a medium containing glucose as the sole source of carbon and 13 per cent D_2O, contain 5.6 atom per cent D in their protein. Using this value in the above calculations rather than 1.62 atom per cent excess as the maximum, we calculate a rate of turnover of $0.33/5.6 \times 100 = 5.9$ per cent per hour.

These data demonstrate that the proteins of the non-growing bacteria are continuously being degraded and resynthesized. The rate is faster than that found in any mammalian tissue. In the logarithmic phase of growth of a bacterial system, this turnover could be masked by the enormous rapidity of protein synthesis. The bulk of the protein in such a system is too young to permit easy measurement of the cumulative results of turnover.

Protein turnover in micro-organisms was recently demonstrated by Mandelstam.[8] Measuring the rate of incorporation of C^{14}-labeled glycine into a leucineless mutant of *E. coli*, he found the turnover rate to be $4^1/_2$ per cent per hour when the bacterial culture was not growing because of the absence of leucine. Somewhat similar results were obtained in bacteria in their growing phase.

Were the premise of Hogness *et al.* valid, that the bulk of the proteins of *E. coli* as well as β-galactosidase is in a static state, then an extrapolation to the mammalian cell might be significant. However, the results reported here, together with those of Mandelstam,[8] clearly demonstrate that, at least in the non-growing *E. coli*, the peptide bonds are being opened and reclosed. The question as to whether or not the dynamic state exists in *E. coli* in exponential growth is not directly touched by our experiments. However, it seems unnecessary, in the absence of direct experimental evidence, to abolish those mechanisms in the slowly growing cell. It is obvious that during exponential growth the rate of protein synthesis is much larger than the rate of degradation. In *E. coli* the synthetic rate seems twenty times larger than the degradative one. Evidently great care might be needed to observe this relatively slow reaction. In the absence of direct evidence to the contrary, the existence of the dynamic state should not be dismissed.

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† Fellow of the John Simon Guggenheim Memorial Foundation, 1957–58.

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*THE TRANSFER OF IRRADIATION-ELICITED INDUCTION IN A LYSOGENIC ORGANISM**

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From studies on the mechanism of induction of lysogenic organisms by ultraviolet irradiation, we arrived at the tentative conclusion that the effect of the irradiation is not direct but rather that it is mediated by some radiation-sensitive metabolite.[1] Recently Northrop has offered a similar hypothesis.[2]

To explore the validity of our hypothesis, we have made numerous attempts to transfer the effects of irradiation from an irradiated lysogenic cell to a non-irradiated one. We have irradiated in the cold inducible lysogenic organisms, disintegrated them by a variety of methods, incubated intact lysogenic cells in the debris, and assayed for induction by the standard techniques. All such attempts at the transferring of induction have proved unsuccessful so far.

If such an irradiation-mediating metabolite does exist, two attributes of it might defeat any attempt at transfer: it might be too unstable; it might not penetrate the barrier of the bacterial cell wall.

It occurred to us that the optimum condition for carrying out such a transfer might be during the conjugation of an irradiated cell with an unirradiated one, provided, of course, that the irradiation does not inhibit conjugation. We report here the transfer of induction from an irradiated lysogenic donor cell to an unirradiated acceptor cell during mating.

The principle of these studies is as follows: A donor organism (with an F^+ sexual orientation), *E. coli* $K_{12} \cdot W \cdot 6 \cdot F^+ \cdot M^- \cdot L_p^+$ is deprived of its essential nutrient, methionine, and is irradiated. Without its essential amino acid, the organism is known to be incapable of producing lambda phage[3] (see Table, col. 5, Expts. 1, 2, 3). The irradiated, methionine-deprived F^+ organism is then admixed with a receptor organism (with an F^- sexual orientation) *E. coli* $K_{12}\ W_{1177} \cdot F^- \cdot Thr^- \cdot Leu^- \cdot B_1^- \cdot L_p^+$ in a medium adequate for the latter but not for the former, and the formation of free phage is assayed.

E. coli $K_{12}\ W \cdot 6 \cdot F^+$ was centrifuged during the logarithmic phase of growth and was washed with medium lacking methionine.[4] It was suspended to a concentration of 10^8 cells/ml in the medium lacking methionine and was irradiated for 180

seconds, which was known to induce over 90 per cent of the cells. The irradiated culture was mixed with *E. coli* K_{12} W_{1177} · F^- at a cell density of 10^8/ml, in a medium which can support the growth of the latter, i.e., containing leucine, threonine, and thiamin, but not the former, i.e., lacking methionine. The mixed cultures were incubated in the dark for 140 minutes. After the incubation, the unlysed cells were eliminated by centrifugation at 5,000 rpm for 20 minutes, and the free λ phage was assayed by plating on the sensitive strain, *E. coli* K_{12} C-600-25, originally isolated by Dr. Appleyard.[5]

In Table 1, Experiment 1, characteristic data are presented. When the irradiated methionine-requiring auxotroph is incubated alone without methionine, the phage yield is less than 10^5/ml. When it is mixed with *E. coli* K_{12} W_{1177} · F^-, in a medium adequate for the growth of the latter the phage yield is 6.3×10^7/ml. The receptor organism alone during the same period produces 2×10^6 phage/ml. Therefore, the yield of phage resulting from the admixture of the two cultures is at least 6×10^7. The burst size of these organisms in synthetic medium, is *ca.* 15; therefore, the number of cells which produced phage can be calculated to be 4×10^6/ml. Since the number of originally irradiated cells in the final mixture is known to be 5×10^7/ml, the effective transfer of induction—a phenomenon to which we shall refer from now on as "cross-induction"—is about 8 per cent.

TABLE 1

CROSS-INDUCTION UNDER DIFFERENT CONDITIONS

No.	Experiment Type	Irradiated Organism No./Ml × 10^{-7})		Irradiation (Sec.)	Background Phage (In 140 Min.)	Unirradiated Organism (No./Ml × 10^{-7})		Background Phage (In 140 Min.)	Total Phage (In 140 Min.)
1.....	Standard F^+ to F^-	A*	5.0	180	$<10^5$	B	5.0	2×10^6	6.3×10^7
2.....	Increased F^+	A	35.0	320	$<10^5$	B	5.0	3×10^6	2×10^8
3.....	F^+ to F^+	A	5.0	180	$<10^5$	C	5.0	9×10^5	1.2×10^6
4.....	F^- to F^+	B	5.0	180	$<10^5$	A	5.0	2×10^6	4×10^6

* The following is the key to the code of the organisms used:
A = *E. coli* K_{12} W_{-6} F^+ · M^- · L_p^+.
B = *E. coli* K_{12} W_{1177} · F^- · Thr^- · Leu^- · B_1^- · L_p^+.
C = *E. coli* K_{12} W_{1817} · F^+ · Thr^- · Leu^- · B_1^- · L_p^+.
All organisms were originally isolated and kindly provided by Dr. Joshua Lederberg.

Since the receptor cells are in excess (they go through 2.3 cell divisions in 140 minutes), a possible enhancement of cross-induction might be achieved only by increasing the ratio of irradiated donor cells to receptor cells. Experiment 2 summarizes such an experiment. The increased dose of irradiation was necessitated by the greater population density. The per cent induction[6] was similar in Experiments 1 and 2.

That conjugation is an essential prerequisite of cross-induction is indicated by the findings in Experiment 3. Here the irradiated donor was admixed with an auxotroph of similar sexual orientation, *E. coli* K_{12} W_{1817} · F^+. The yield of phage was only slightly above the background, indicating only a slight cross-induction, which may actually be due to the presence of a small percentage of F^--type organism in the unirradiated culture.

The negative findings in the homosexual crossing also serve to eliminate mechanisms other than cross-induction as the basis of the observed phenomenon. It eliminates the possibility that the increased phage formation is due to the cross-

feeding of methionine to the irradiated donor. As further evidence of this, we have incubated irradiated *E. coli* K_{12} W $\cdot_6 \cdot$ F^+ in sterile culture fluids in which the recipient cells, *E. coli* K_{12} $W_{1177} \cdot F^-$ had grown to late logarithmic growth phase. No enhancement of phage formation could be detected, indicating that there had been no leakage of methionine into the medium by the auxotroph, which is capable of synthesizing this amino acid.

The negative findings in the homosexual crossing also rule out the possibility that the enhanced phage formation is due to the irradiation of the medium itself.

That cross-induction is unidirectional, going only from F^+ to F^- organisms, is indicated by Experiment 4. Here *E. coli* K_{12} $W_{1177} \cdot F^-$ was irradiated in a medium lacking threonine, leucine, and thiamine and was admixed with *E. coli* K_{12} W $\cdot_6 \cdot F^+$ in a complete medium for the latter. No excess phage formation was observed.

Finally, that cross-induction is dependent on the irradiation of the donor cell and is not merely some form of zygotic induction[7] was evidenced by the admixture of the unirradiated F^+ with F^- cells, which yielded no increase in phage formation.

There are two different mechanisms which could account for cross-induction. The prophage altered by the irradiation, along with other genetic material of the donor cell, may enter *in toto* during conjugation into the receptor cell; or some other irradiation-altered molecule may penetrate and initiate induction in the receptor cell as it would in the originally irradiated cell. We can offer two different lines of evidence indicating that it is the second mechanism which accomplishes cross-induction.

1. Cross-induction is still possible under conditions which destroy a cell's capacity for genetic recombination. Such dichotomy was established by exposing the donor F^+ organisms to massive doses of irradiation (Expts. 1 and 2, Table 2). When *E. coli* K_{12} W $\cdot$ 6 $\cdot$ F^+ was exposed to irradiation for 400 and 500 seconds instead of the usual 180 seconds and was mixed with the receptor cells, it is evident from the data that cross-induction was greater than with the smaller dose of irradiation. At the same time, such heavily irradiated F^+ organisms were mixed with the F^- organisms, and search was undertaken for genetic recombination by the standard technique for the demonstration of reversion to prototrophy. It was found that the organisms irradiated for 400 seconds produced 1 or 2 prototrophs from 10^7 cells and that those irradiated for 500 seconds produced none. In identical controls with cells irradiated for 180 seconds about 50 and with unirradiated cells, about 100 prototrophs arose from 10^7 cells. Therefore, massive irradiation destroys the mechanisms for genetic recombination but not for cross-induction.

2. If cross-induction could be demonstrated between an irradiated F^+ cell, which itself is not lysogenic, i.e., lacks a prophage, and a lysogenic F^- cell, then the possibility of prophage transfer would be completely eliminated. In Experiment 3, Table 2, such a phenomenon is demonstrated. *E. coli* K_{12} $W_{1485}F^+ \cdot L_p^-$ (a non-lysogenic organism) was irradiated and was admixed with *E. coli* K_{12} $W_{1177} \cdot F^- \cdot L_p^+$. The thirty-fold increase in phage formation could stem only from the transfer of the effects of the irradiation by a carrier other than the prophage. (The admixture of unirradiated non-lysogenic F^+ cells with unirradiated lysogenic F^- cells produced no phage over the background.)

The findings reported here demonstrate that induction and phage formation in a lysogenic organism can be separated into sequential events in two different cells.

The initial impact of the irradiation does not appear to be at the locus of the prophage but rather at some component of the cell other than the genetic material.

TABLE 2

CROSS-INDUCTION UNDER CONDITIONS PRECLUDING PROPHAGE TRANSFER

No.	Experiment Type	Irradiated Organism (No./Ml $\times 10^{-7}$)	Irradiation (Sec.)	Background Phage (In 140 Min.)	Unirradiated Organism (No./Ml $\times 10^{-7}$)	Background Phage (In 140 Min.)	Total Phage (In 140 Min.)
1	Overirradiated	A* 5.0	400	$<10^5$	B 5.0	2×10^6	1.2×10^8
2	Overirradiated	A 5.0	500	$<10^5$	B 5.0	2×10^6	9×10^7
3	Non-lysogenic donor	D 22.0	400	None ($<10^4$)	B 5.0	8.7×10^5	3.2×10^7

* Micro-organisms A and B are the same as in Table 1. D = *E. coli* K_{12} W_{1485} · F^+ · L_p^- (non-lysogenic).

The substance altered by the radiant energy, which then mediates the effects of the irradiation, is stable enough to be successfully transferred from cell to cell during conjugation, thus offering a promise that a chemical search for such a substance may not be barren. Moreover, since ultraviolet irradiation is but one of the agents capable of inducing lysogenic organisms, the exposure to mutagens and carcinogens being the others, it will be of interest to ascertain whether cross-induction is possible with those agents as well, and to establish whether any intermediates exist in the mechanism of action of mutagenic or carcinogenic agents.

It is a pleasure to acknowledge our indebtedness to Professor Francis J. Ryan, of this university, for fruitful discussions.

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*EVIDENCE FROM TETRAD ANALYSIS FOR BOTH NORMAL AND ABERRANT RECOMBINATION BETWEEN ALLELIC MUTANTS IN NEUROSPORA CRASSA**

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Evidence accumulated during the past few years indicates that aberrant recombination occurs in crosses of allelic mutants in organisms such as *Neurospora* and yeast in which the presence of chromosomes exhibiting regular meiotic behavior leads to the expectation of conventional genetic mechanisms. Early observations in *Neurospora* suggesting atypical behavior came from studies by Giles[1, 2] on the origin of prototrophs obtained in random ascospore platings from crosses between allelic inositol mutants carrying linked markers on either side of the inositol locus. Although the origin of inositol-independent isolates exhibited a positive correlation with crossing over between adjacent markers—the majority of such isolates occurring in one crossover class—the reciprocal crossover class and the two parental classes with respect to the markers were also represented in the progeny. Evidence that atypical segregation rather than negative interference is involved in a similar situation in *Neurospora* has been provided by Mitchell[3, 4] on the basis of an analysis of tetrads from a cross of two pyridoxine mutants. These results indicated that asci containing pyridoxine-independent isolates did not contain the reciprocal double mutant expected on the basis of conventional crossing over. Similar results from tetrad studies have been reported in yeast by Lindegren[5, 6] and by Roman,[7] and this phenomenon has been referred to by Lindegren[5] as "gene conversion."

Although the phenomenon of "gene conversion" appears well established in at least some of these cases, the possibility clearly remains that both conventional crossing over as well as atypical segregation may occur in such crosses. In an apparently comparable situation in *Aspergillus*[8] the results are different from those in *Neurospora*. In this instance, from a cross of two adenine mutants, the expected double mutant has been recovered (although not in tetrads, but by mitotic rather than by meiotic recombination), even though the distribution of adjacent markers in adenine prototrophs from random ascospore isolations from the same cross suggests aberrant recombination. Thus it would appear significant to determine from tetrad data whether both "gene conversion" and conventional crossing over can occur in a cross of the same two mutants. Evidence on this problem is provided in the present studies from tetrad analyses of crosses of two pantothenic acid-requiring mutants in *N. crassa* carrying appropriate linked markers.

Materials and Methods.—The pantothenicless mutants used in these studies were obtained by means of several filtration-concentration experiments employing the technique of Woodward *et al.*[9] and utilizing untreated, X-irradiated, or ultraviolet-irradiated macroconidia of three wild-type strains of *N. crassa:* 74A (obtained from Dr. Patricia St. Lawrence), 5.5A, and 3.1a—the latter two strains having originated from further inbreeding experiments with 74A. As will be indicated

in detail later, these pantothenicless mutants are physiologically (by heterocaryon tests), genetically (by crossing analyses), and biochemically (on the basis of precursor utilization) different from the previously known pantothenicless mutant 5531 (Barratt *et al.*[10]). Hence the mutant locus in strain 5531 (located in linkage group IV) is now termed the *pan-1* locus (also designated as "group A pantothenicless mutants" in this paper), and these new pantothenicless mutants (which have been located in linkage group VI) are designated as mutants at the *pan-2* locus (also referred to herein as "group B pantothenicless mutants"). In later discussions of these mutants, the shortened designation "pan mutants" will be employed. The other mutant strains used in the genetic analyses were *ad-1* (adenine—3254), *tryp-2* (tryptophan—75001), and *ylo* (yellow).[10]

The growth medium used throughout was Fries minimal[11] with appropriate biochemical supplementations. Crosses were made on supplemented Westergaard's synthetic crossing medium,[12] or on a modified low-sulfate Westergaard's with $MgSO_4$ replaced by 0.4 gm/l $MgCl_2$.[13] The only sulfate source in this medium is adenine sulfate (used at a concentration of 50 μg/ml). The technique described by Newmeyer[14] was used in all random ascospore platings. Heterocaryon tests were made with conidial suspensions of the strains on minimal agar Petri plates in the manner described by de Serres.[15]

Biochemical Relationships of the Mutants.—The previously known *pan-1* mutants in *Neurospora* were found to be blocked in the terminal reaction in pantothenic acid synthesis in which β-alanine and pantoic acid are coupled.[16] Preliminary tests with the *pan-2* mutants indicated that growth would occur on pantoyl lactone (the lactone of pantoic acid) as well as on calcium pantothenate. Since the *pan-2* mutants differed biochemically from the *pan-1* mutants, their position in pantothenic acid synthesis had to be determined.

On the basis of the scheme for pantothenic acid synthesis in *Escherichia coli* described by Maas and Vogel,[17] growth-stimulation tests utilizing the probable precursors of pantothenic acid—ketovaline, ketopantoic acid, and pantoic acid—were made with *pan-2* mutants. The results indicate that all the *pan-2* mutants are blocked in the same step in pantothenic acid biosynthesis—the conversion of ketovaline to ketopantoic acid—since all mutants will grow on ketopantoic acid, pantoic acid, and pantothenic acid, but not on ketovaline.

Intra-group Heterocaryon Relationships of the Pan-2 Mutants.—The assumption was made in early investigations with *Neurospora* that complementation between two phenotypically similar biochemical mutants resulting in growth of a heterocaryon on unsupplemented media indicates non-allelism, whereas non-complementation establishes two mutants as alleles.[18] All the 37 *pan-2* mutants show a positive heterocaryon response with a genetically and biochemically different mutant (mutant *A1*) at the *pan-1* locus, and it was anticipated on the basis of their biochemical similarity that *pan-2* mutants would exhibit no complementation with one another. However, preliminary tests with all possible combinations by twos of the first 7 *pan-2* (group B) mutants indicated that two of the mutants, *B3* and *B5*, formed a pantothenic acid–independent heterocaryon (bicaryon[19]). Additional heterocaryon responses with 7 more B mutants were found in tests employing all the *pan-2* mutants in all possible combinations by twos. Such heterocaryons formed between mutants assumed to be allelic are termed "intra-group hetero-

caryons." The pattern of complementation of the mutants established three major groups: (1) mutants *B3*, *B7*, *B19*, *B20*, *B25*, and *B36*, which complement with (2) mutants *B5*, *B23*, and *B28*, and (3) most of the remaining mutants, which exhibit no complementation responses. Several of these combinations (*B3* with *B23* and *B28*; *B7* with *B23* and *B28*) are partially temperature-sensitive in exhibiting reduced growth at 35° C. In addition, the heterocaryon between *B3* and *B5* is completely temperature-sensitive, in that it does not grow on minimal at 35° C. This fact has been particularly useful in the subsequent genetical analyses.

Genetic Relationships of the B Mutants.—The biochemical and heterocaryon studies just described indicated that the B mutants were distinct from the previously known *pan-1* mutants in *Neurospora*. Despite peculiarities in the pattern of heterocaryon complementation, the biochemical results suggested that the B mutants were probably allelic in the conventional sense. However, it was necessary to provide genetic evidence on this point.

To establish the linkage relationships of the B mutants, one mutant, *B3*, was crossed to markers in each of the seven linkage groups. Random isolations indicated that *B3* was associated with *ylo*, *ad-1*, and *tryp-2* in linkage group VI. All the *pan-2* mutants were then crossed to a common stock, *ad-1 tryp-2*, and by random ascospore platings were found to be between these two markers. There were a few instances in which abnormal linkage with one of the markers was noted. Additional tests established that the position of the *pan-2* locus with respect to the three other loci utilized in linkage group VI is as follows: *ylo*, *ad-1*, *pan-2*, *tryp-2*. These results showed that all 37 *pan-2* mutants were located in the same general region of linkage group VI but also indicated that a more detailed genetic analysis was necessary to establish their precise relationships.

Tetrad Analyses of B3 × B5 Crosses.—In the initial studies with the first seven B mutants, only *B3* and *B5* formed an intra-group heterocaryon. Additional heterocaryon tests with the other thirty mutants indicated that most of the mutants which gave an intra-group heterocaryon response could be classified as being generally similar to either *B3* or *B5*. Hence these two complementing mutants were selected for further study. This study consisted of tetrad analyses to obtain information on the inheritance of the heterocaryon complementation pattern and possible evidence for exceptional behavior in segregation or in recombination at the *pan-2* locus.

Two marked crosses of similar genotypes (*ylo ad-1*$^+$ *B3 tryp-2* × *ylo*$^+$ *ad-1 B5 tryp-2*$^+$) were utilized in the tetrad analyses. However, in the first cross the *B3* parent was not heterocaryon-compatible with the B mutant testers, and hence segregation for *B3* and *B5* could not be examined. A more extensive analysis was made in the second cross, since in this cross both parental stocks were heterocaryon-compatible with the original *B3* and *B5* mutants. Thus segregation of *B3* and *B5* could be followed in the second cross by testing the heterocaryon response of the isolates.

Seventy-two complete tetrads (at least one of each spore pair germinating) were obtained in the first cross (out of 100 asci isolated), and 856 complete tetrads (encluding 11 exceptional asci to be discussed later) from 1,299 asci isolated in the second cross.

Since more complete data are available from the second cross, the linkage rela-

tionships of the *pan-2* locus with the adjacent markers as determined from "normal asci" (those having two 2 *B3* and 2 *B5* spore pairs) in this cross are given in Table 1.

TABLE 1

RESULTS OF TETRAD ANALYSES FROM 856 NORMAL COMPLETE ASCI
(Second Cross: *ylo ad*$^+$ *B3 tryp* × *ylo*$^+$ *ad B5 tryp*$^+$)

CLASSIFICATION OF ASCI	NO. ASCI WITH RECOMBINATION IN THE INTERVALS			
	ylo-ad	*ad-centromere*	*centromere-pan*	*pan-tryp*
Parental ditype	826	851	827	723
Non-parental ditype	0	1	0	1
Tetratype	30	4	29	132
Per cent crossing over	1.7	0.2	1.7	7.7

The results indicate the order and map distances of the mutants to be as follows: *ylo* 1.7 *ad-1* 0.2 centromere 1.7 *pan-2* 7.7 *tryp-2*. All complete asci in the second cross were tested for their heterocaryon responses with *B3* and *B5* testers of the appropriate sex. In all but three asci (to be discussed later) having only mutant isolates, a regular 2:2 segregation of *B3*:*B5* heterocaryon-complementing types was observed. Furthermore, a given type of complementation exhibited linkage with adjacent markers as anticipated on the basis of prior crosses of both *B3* and *B5* mutants with *pan-2*$^+$ strains carrying linked markers. These results indicate that the pattern of heterocaryon response is characteristic of each *pan-2* mutant and that this response cannot be attributed to genetic modifiers at other loci, unless these are exceedingly closely linked to the *pan-2* locus.

Analysis of Asci with Exceptional Segregation Patterns.—On the basis of conventional genetic mechanisms, it was anticipated that all asci from these two crosses would exhibit segregation such that two *B3* and two *B5* spore cultures would be present. However, exceptional segregations were noted in eleven asci (one from the first cross and ten from the second). The genotypes of these asci are shown in Table 2, and the over-all tetrad results with respect to segregation types are summarized in Table 3. The *pan-2* genotype—whether *B3*, *B5*, or *B5 B3*—was determined initially by heterocaryosis and later confirmed by backcrossing to both the *B3* and the *B5* parents.

In two asci, Nos. 21 and 1142, there is an irregular order of isolates, indicating either nuclear passing or, probably more likely, especially in the case of ascus 21, isolation errors. The constitution of ascus 365 is more difficult to interpret because spore cultures 365.7 and 365.8 do not agree in genotype. It appears probable that spore culture 365.8 does not belong with this ascus and constitutes an isolation error, and such will be assumed in further discussion of this ascus. The possibility that the wild-type spore (culture 365.7) arose as a result of post-meiotic reversion was tested by plating conidia. The failure to recover *pan-2* isolates may be taken as evidence against this possibility.

In two of the asci (Nos. 400 and 1167—type 1 of Tables 2 and 3) a wild-type culture (in both instances of the same crossover type with respect to the linked markers) was present, with the reciprocal crossover type testing as a double *B5 B3* mutant. In addition, one of these asci (1167) contained the two single mutant parental types. The second ascus (400) had one parental type present, and it appears reasonable to assume that the spore pair missing in this ascus was the other parental type. Thus these two asci appear to be instances of conventional cross-

TABLE 2

GENOTYPES OF ISOLATES FROM EXCEPTIONAL ASCI AS DETERMINED BY HETEROCARYON AND CROSSING TESTS*

DESIGNATION	ASCUS No.	DISTAL 1	2	3	ORDER OF SPORES IN ASCI 4	5	6	7	PROXIMAL 8
Type 1	400	y a	y a	...	...	ad B5 B3 t A	ad B5 B3 t A	y B3 t A	y B3 t A
	1167	y B3 t A	y B3 t A	y A	y A	ad B5 B3 t a	ad B5 B3 t a	ad B5 a	ad B5 a
Type 2	21†	y B3 t A	ad B5 a	ad B3 t a	ad B3 t a	y A	y A	ad B5 a	y B3 t A
	145	y A	y A	y B3 t A	y B3 t A	ad B3 t a	ad B3 t a	ad B5 a	ad B5 a
	1142	y B3 A	y t A	y B3 A	y t A	ad B5 a	ad B5 a	ad B3 t a	ad B3 t a
	1200	ad B5 a	ad B5 a	ad B3 t A	ad B3 t A	y A	y A	y B3 t a	y B3 t a
Type 3	291	ad B5 a	ad B5 a	...	...	y B5 a	...	y t A	...
	365	ad B5 a	ad B5 a	ad B5 a	...	y B3 t A	y B3 t A	y t A	y B3 t A‡
Type 4	1029	ad B5 A	ad B5 A	ad B3 A	ad B3 A	y B3 t a	y B3 t a	y B3 t a	y B3 t a
	1109	ad B3 A	ad B3 A	ad B5 a	...	y B3 t A	y B3 t A	y B3 t a	y B3 t a
	1208	y B3 t A	y B3 t A	y B3 t a	y B3 t a	ad B3 A	ad B3 A	ad B5 a	ad B5 a

* Key: y = ylo; ad = adenineless; t = tryptophanless; A or a = mating type; B3 or B5 = *pan-2* B mutants.
† This ascus from the first cross, the other ten from the second cross.
‡ This isolate presumably does not belong with this ascus; see text for further discussion.

ing over at the four-strand stage in which two parental non-crossover and two reciprocal crossover products result. On the basis of these two asci the linear order of *B3* and *B5* with respect to the adjacent markers is indicated as *ylo, ad-1, B5 B3, tryp-2*. The distance between *B5* and *B3* is 0.1 crossover unit (2 asci in 939).

In contrast to the two preceding asci, there was no evidence in the six additional asci containing wild-type spore cultures for the expected reciprocal double *pan-2* mutant. Rather, in these asci, either one or the other parental type was represented twice. In four asci having two *B3* mutants (type 2), crossing over had occurred between the markers such that in each instance the wild-type spore culture represented either a single or a double (ascus 1142) crossover chromatid. In the two asci having two *B5* mutants (type 3), crossing over had occurred between the markers in ascus 291 on the proximal side of the *pan-2* locus, but the wild-type spore culture was a non-crossover type, while in ascus 365 there was no evidence of crossing over. However, it should be noted that a two-strand double crossover on either side of the *pan-2* locus would not be detectable in either of these asci.

The remaining exceptional asci (1029, 1109, and 1208—type 4) were distinct from the other eight in lacking wild-type spore cultures. Although all spore cultures were pantothenic acid-requiring, the segregations were irregular, in that each of the three asci contained one *B5* and three *B3* types. In each instance two of the *B3* cultures and one *B5* culture were non-crossover types, while the third *B3* was a double crossover.

These exceptional asci were further characterized by checking their heterocaryon responses with other intra-group testers and by backcrossing to *B3* and to *B5*. The isolates which tested as *B3* or *B5* in the original heterocaryon tests of the asci responded in the same manner with the other group *B3* and group *B5* testers. Also the two *B5 B3*

double mutants, originally identified as heterocaryon-negative with both *B3* and *B5*, tested similarly with the other B group testers. Additional tests of the apparent double mutants indicated no complementation with either *B3* or *B5* even in forced heterocaryons.

TABLE 3

SUMMARY OF TYPES OF SEGREGATIONS IN CROSS OF *pan-2* MUTANTS *B3* × *B5*

Types of Asci	No. Asci
Normal segregation	
2 *B3*:2 *B5*	856
Exceptional segregations	
Type 1	
1*B5*:1 *B3*:1 WT:1 *B3 B5*	2
Type 2	
1 WT:1 *B5*:2 *B3*	4
Type 3	
1 WT:1 *B3*:2 *B5*	2
Type 4	
1 *B5*:3 *B3*	3

Genetic analyses of the isolates from exceptional asci consisted of backcrosses of such isolates to both *B3* and *B5* parental types carrying appropriate linked markers. These crosses were tested by random ascospore platings for the recovery of true wild types (pantothenic acid–independent isolates). True wild types could be easily distinguished from pseudo-wild types, which also occur, since the latter give rise to temperature-sensitive heterocaryons which do not grow in the absence of pantothenic acid at 35° C. Wild types were obtained only from crosses involving isolates testing phenotypically as *B3* × *B5*. No wild types were obtained from presumptive selfings of either *B3* or *B5* or from crosses of the presumptive double *B5 B3* isolates with either *B3* or *B5*. Furthermore, the distribution into crossover and non-crossover categories (with respect to adjacent linked markers) of the wild types derived from these backcrosses was, in general, similar to that obtained in the original *B3* × *B5* cross (Table 4). Further genetic tests in which one *B5 B3*

TABLE 4

CLASSIFICATION OF PAN PROTOTROPHS OBTAINED IN RANDOM ASCOSPORE ISOLATIONS*

TYPE OF CROSS	TOTAL VIABLE COLONIES	NO. PAN PROTOTROPHS	PER CENT PAN PROTOTROPHS IN FOUR CLASSES			
			Non-crossover		Crossover	
ylo ad$^+$ *B3 tryp* (= P1) × *ylo*$^+$ *ad B5 tryp*$^+$ (= P2)			P1	P2	*ylo*$^+$	*ad tryp*
B3 × *B5* (parental cross)	129,256	212	38.8	13.8	40.0	7.1
B3 × 145.7	70,806	185	28.6	21.6	41.6	8.1
B3 × 1208.7	125,100	162	27.1	25.3	40.1	7.4
ad B3 tryp (= P1) × *ad*$^+$ *B5 tryp*$^+$ (= P2)			P1	P2	*ad* $^+$	$^+$ *tryp*
21.4 × *B5*	34,268	55	21.8	34.5	38.1	5.4
ylo B3 tryp (= P1) × *ylo*$^+$ *B5 tryp*$^+$ (= P2)			P1	P2	*ylo* $^+$	$^+$ *tryp*
145.4 × *B5*	76,698	180	27.3	22.9	41.3	8.3

* Data for the parental cross and for representative crosses (to either *B3* or *B5*) of pan isolates from the exceptional asci.

double was crossed to wild type indicated that it is possible to reisolate from such a cross both *B3* and *B5* single mutants. Thus all these crossing results serve to confirm by genotypic tests the phenotypic determinations derived from the heterocaryon tests and substantiate the conclusion that four types of exceptional asci have been obtained in the tetrad analyses.

Discussion.—The evidence from tetrad analyses of crosses of the pan mutants (*B3* × *B5*) supports the view that both conventional and aberrant recombination can occur between these two mutants.

The simplest interpretation of the two asci of type 1 is that these have resulted from conventional reciprocal crossing over in the four-strand stage between the two pan mutants (which may be represented as *B5 B3*$^+$ and *B5*$^+$ *B3*), giving rise to one pan-independent (*B5*$^+$ *B3*$^+$) and one reciprocal double mutant isolate (*B5 B3*), accompanied by the two non-crossover parental single mutants. The recovery in a single chromosome of a double mutant identified as such both phenotypically and genotypically (and from which it has been possible to recover by subsequent recombination both the two original single mutants) clearly establishes the separability of the two *pan-2* mutants by the criterion of recombination. Furthermore, the marker relationships in these two asci permit the establishment of a linear order of the two mutants, with *B5* proximal to *B3*.

The additional separability of these two mutants on a functional basis is indicated by their biochemical complementation, which results in growth in the absence of pantothenic acid when the two mutants are tested in a heterocaryon. Present evidence indicates that both mutants are blocked in the same step in pantothenic acid synthesis and presumably lack the same enzyme. Hence, although the enzymatic evidence has not yet been obtained, the probability appears high that these mutants are similar to mutants which lack adenylosuccinase but exhibit heterocaryon complementation associated with restored enzyme activity.[19] Thus on functional evidence these two pantothenic mutants may be considered to have resulted from independent mutations in two distinct "cistrons."[20] On this basis, the occurrence of conventional recombination between them is not surprising.

However, the three additional exceptional types of tetrads indicate that apparently aberrant recombination between these two mutants may also occur. Before attempting to interpret such tetrads in terms of novel genetic mechanisms, the possibility must be considered that such tetrads may be explained on the basis of conventional, albeit unusual, meiotic behavior. The most likely explanation of this type for the exceptional segregations observed appears to involve the possible occurrence of triploid, or especially of trisomic, nuclei at meiosis. However, preliminary cytological studies of asci from *B3* × *B5* crosses indicate the regular occurrence of seven bivalents at MI of meiosis and of only seven chromosomes at all first post-meiotic mitoses examined. Additionally, there is strong genetic evidence against a heteroploidy hypothesis. In particular, the segregation of adjacent linked markers in all the asci, both normal and exceptional, has been regular rather than aberrant. Also, there are no instances of 4:0 or 0:4 segregations for markers or for the pan mutants, as would be anticipated if extra chromosomes were segregating. Despite this general evidence against heteroploidy, it is still theoretically possible to explain the exceptional segregations on the basis of trisomic

behavior. However, such interpretations require apparently highly improbable sequences of trivalent crossing over, non-disjunction at AI, and post-AI chromosome loss. On the basis of all the above evidence the heteroploidy hypothesis has been discarded as an explanation for the aberrant tetrads.

Unequal crossing over has been considered as another conventional mechanism whereby the last three types of aberrant tetrads might arise. However, selfings of either *B3* or *B5* do not give rise to pan prototrophs. In addition, in a genetic analysis of the wild types from one type 2 ascus, crosses were made between the original wild-type culture and the reciprocal crossover *B3* culture from the same ascus. In the latter intra-ascus cross, heterocaryon tests of a substantial number of pan isolates failed to give any evidence that *B5* types could be recovered. This result appears to eliminate the possibility that such wild types, although phenotypically wild, may contain the *B5* element in an unusual arrangement detectable only by such an intra-ascus cross.

The absence of evidence for conventional, even though unusual, genetic mechanisms makes it probable that the three types of aberrant tetrads have resulted from novel recombination mechanisms which have been referred to by such terms as "gene conversion,"[5] "copy-choice,"[21] "transmutation,"[22] and "transreplication."[23] The present study differs from any previous ones in that in this instance it is possible to follow the simultaneous segregation of two close-linked mutants separable by conventional crossing over and phenotypically distinguishable when alone or together. On this view, either the proximal *B5* or the distal *B3* or both mutants simultaneously can exhibit atypical 3:1 segregations.

Although conventional reciprocal crossing over in a diploid nucleus cannot account for the aberrant tetrads, the evidence from the adjacent linked markers indicates that the occurrence of such types is positively correlated with crossing over in the vicinity of the *pan-2* locus. In four of the six asci having a pan-independent isolate with no accompanying reciprocal double mutant (types 2 and 3), this isolate is a crossover type. This result agrees with data on pan prototrophs obtained from random ascospore isolations from *B3* × *B5* crosses, which also indicate a marked positive correlation between the origin of such types and adjacent marker recombination.

In the present tetrad data the types of aberrant segregations may be interpreted even more specifically in terms of the pattern of adjacent crossing over. Thus, in all four asci of type 2 (1WT:2*B3*:1*B5* and hence segregating 3*B5*$^+$: 1*B5*, but 2*B3*:2*B3*$^+$ on the basis of two separable mutants) crossing over has occurred between the *pan-2* locus and the centromere, thus adjacent to *B5*, the mutant exhibiting "conversion." (In one ascus, No. 1142, crossing over has also occurred between the *pan-2* locus and the *tryp-2* marker such that this pan prototroph is actually a double crossover.) The two asci of type 3 (1WT:2*B5*:1*B3*, and hence 2*B5*$^+$:2*B5*, but 1*B3*:3*B3*$^+$) are apparently non-crossover types. However, these two asci may also be interpreted as two-strand double crossover tetrads in the *pan-2–tryp-2* interval, with one crossover occurring closely adjacent to *B3*, the mutant exhibiting "conversion." Support for this interpretation comes from the fact that the distance between *B3* and the adjacent distal marker (*tryp-2*) is over four times that between *B5* and the adjacent proximal marker (the centromere). Additionally, "conversion" of *B3* associated with a single crossover event in the

B3-tryp-2 interval has been obtained in a cross of a *B5 B3* double with wild type (Case, unpublished).

Finally, the three asci of type 4 must be considered. These asci contain no pan-independent isolates but rather have 3*B3*:1*B5* isolates. Thus they appear to be instances in which two simultaneous "conversions" took place—in one instance of *B5* to *B5*$^+$, and in the other of *B3*$^+$ to *B3*. With respect to markers, in all three asci two of the *B3*'s and the one *B5* are non-crossovers, whereas one *B3* is a double crossover, one crossover having occurred in the centromere-*pan-2* interval, and the other in the *pan-2–tryp-2* interval. Although it is impossible in these asci to determine which *B3* isolate contains the "converted" mutants, a reasonable interpretation appears to be that this isolate is, in each instance, the double crossover type and hence that two simultaneous crossovers, one adjacent to *B5* the other to *B3*, have occurred in association with these "conversion" events.

The preceding discussion suggests a precise positive relationship between adjacent conventional crossing over and aberrant segregations. Such a relationship does not, however, provide a mechanism to explain these segregations. Aberrant tetrad ratios may be interpreted on the basis of either of two principal hypotheses. One of these involves "gene conversion" in the sense of directed gene mutation occurring at meiosis.[2, 5, 6] The other involves a "copy-choice" or "transreplication" mechanism, in which one of the two newly formed chromatids is assumed to replicate, in the region showing aberrant segregation, using its homologue as a template.[21] The present results are taken to favor the second of these two hypotheses for the following reasons: (1) In this study only 3:1 or 1:3 and no 4:0 or 0:4 segregation ratios have been obtained. The latter ratios would presumably be anticipated on the "conversion" hypothesis, since directed mutation might affect a given strand either before or after replication, whereas, on the simplest copy-choice mechanism, the two parental mutants types should always be present. (2) The *B3* mutant is very stable in conidia and has not been shown to be capable of back-mutating to wild type either spontaneously or following irradiation. Despite this fact, *B3* reversions occur at meiosis, suggesting an origin other than mutational. (3) In all instances of conversion, the resulting isolates, whether wild type (type 2 and 3) or mutant (*B3* mutants in type 4) have been indistinguishable from the original types (either the pan *B3* parental type or 74A wild type) by all available criteria (e.g., on the basis of heterocaryon complementation patterns and mutational stability). Although these results are not incompatible with a mutational hypothesis, provided that such mutations are very specifically directed, they appear more reasonably interpreted on a copy-choice mechanism. (4) The occurrence of exceptional asci of type 4 (3*B3*:1*B5*) appears to provide especially strong evidence for a copy-choice rather than a mutational mechanism. If mutation is involved, these asci require for their origin the simultaneous occurrence in one *B5* mutant of two mutations, one from mutant to wild type (the *B5* element mutating to *B5*$^+$), the other from wild type to mutant (the *B3*$^+$ element mutating to *B3*). On a copy-choice mechanism, however, it is sufficient to assume that the replication of the *B5* strand involves a copying of the two adjacent elements (*B5*$^+$ and *B3*) from the *B3* strand as a "single event."

The possibility has also been considered that the observed aberrant segregations may involve a phenomenon similar to transduction. On this interpretation, a

small segment (including either the *B5*$^+$, the *B3*$^+$, or the entire *B5*$^+$ *B3* region) of one parental chromosome (presumably originating by an extra replication) is subsequently incorporated, after synapsis with its homologous region in the other parental-type chromosome, by a mechanism equivalent to a two-strand double crossover. On this view, unless incorporation occurs almost immediately after meiosis, the resulting phenotypically wild-type cultures should give rise to heterocaryons in which mutant nuclei are present. In addition, such wild types should be temperature-sensitive, as is the *B3 B5* heterocaryon. However, no mutant nuclei have been detected in such wild-type cultures either by conidial platings or by crossing, nor are the wild types temperature-sensitive.

The possibility of a prolonged synaptic association without incorporation, resulting in the maintenance of a homocaryotic wild-type phenotype, was also considered. Evidence against this situation was obtained from radiation experiments, which indicated that such wild-type cultures behave essentially like the original wild-type strain both quantitatively and qualitatively with respect to the induction of new pan mutants.

Additional general evidence against any transduction hypothesis involving heterogenote synapsis[21] comes from the existence of asci of type 4 (which do not contain any wild-type cultures but exhibit 3:1 segregations for both mutants), since the association of a *B5*$^+$ *B3* segment with a *B5* chromosome, as would be initially required in the origin of such types, should give a culture phenotypically wild type rather than mutant.

If a type of copy-choice mechanism is indeed responsible for the observed aberrant segregations, the relationship of this process to adjacent conventional crossing over remains to be determined. It may well be that synapsis typically occurs in localized short chromosomal segments in which several recombinational events can occur.[8] If this is so, however, the present evidence indicates that these are not all reciprocal recombinational events but that both reciprocal and nonreciprocal mechanisms are involved. Indeed, both types may be part of a general recombination mechanism, the details of which remain to be elucidated. For example, any such general mechanism will have to reconcile copy-choice interpretations with recent evidence[24] that chromosome duplication (on the basis of DNA measurements) has occurred prior to the initiation of meiosis. On the simplest view, this evidence appears to require that synapsis and copy-choice mechanisms operate prior to meiosis. How such a requirement can be made compatible with conventional views regarding the sequence of events at meiosis is not clear at this time.

With reference to the problem of "negative interference," the present results indicate clearly that this phenomenon, as usually interpreted,[8, 25] is not associated with the origin of pan-independent wild types in these tetrads. In only two asci (type 1) is there evidence for conventional recombination (on the basis of the simultaneous recovery of the reciprocal double mutant), and in both of these the resulting pan-independent isolates are single crossovers. In other asci containing pan-independent isolates (types 2 and 3) the absence of the reciprocal double is taken to indicate that such types have not arisen by conventional crossing over. As has been indicated, however, these asci furnish evidence for an association between orthodox crossing over in the vicinity of the *pan-2* locus and the origin of pan proto-

trophs. Thus there is a positive correlation between adjacent reciprocal and apparently non-reciprocal recombination.

The hypothesis developed earlier assumed that a copy-choice event involving either one or the other of the pan mutants (to give a pan prototroph) is always associated with either proximal or distal adjacent reciprocal crossing over. On this basis, all pan prototrophs should be single crossover types indistinguishable with respect to markers from similar types arising by conventional reciprocal crossing over between the two *pan-2* mutants (*B5* being proximal to *B3*). However, the tetrad data themselves indicate that this is not always the case, since in the two asci of type 3, the pan prototrophs are non-crossovers. Hence it follows either that the hypothesis just mentioned is not correct in assuming that a copy-choice event is always associated with adjacent conventional crossing over or that multiple conventional crossover events (negative interference) tend to occur with an unexpectedly high frequency, at least in the *pan-2–tryp-2* interval. The evidence from random ascospore isolations indicates also, on the basis of much larger numbers of pan prototrophs, that, although the expected single crossover class is the most frequent, both parental classes are found, as is the other single crossover class. Thus negative interference may be involved in the origin of the parental classes and the second crossover class. At present, however, the simpler assumption appears to be that a copy-choice event is not always associated with adjacent crossing over. A decision between these alternatives may be possible if more closely adjacent markers become available, especially on the distal side of the *pan-2* locus.

The problem of interpreting the relative frequencies of the four classes of pan prototrophs with respect to adjacent markers still remains. In the tetrad results, pan prototrophs occurred in only two classes—three in the *B3* parental and 5 in one crossover class. In random spore data, all four classes are represented, but there, is, in general, a marked asymmetry such that the two classes found in the tetrad analysis are in excess. In the random spore platings the asymmetry of the two parental classes may arise from an inherently greater probability that one of the two mutants (*B3*) will be involved in a copy-choice event unassociated with adjacent crossing over. An additional possibility involves associated multiple crossover events, as has already been pointed out. The asymmetry of the two crossover classes is presumably due in part to the fact that only one of these contains pan prototrophs arising by conventional crossing over. On the basis of the tetrad results, however, this fact alone cannot account for the magnitude of the observed excess in this class. An additional factor appears to be one already suggested—a positive correlation between adjacent conventional crossing over and the occurrence of a copy-choice event. It should be noted that this asymmetry permits the utilization of the two crossover classes from random data in establishing a linear order for the two mutants, despite the fact that the majority of individuals in the class in excess presumably have not arisen by conventional crossing over between the two mutants. It is evident, however, that the over-all frequency of pan prototrophs determined from random spore data does not give a correct estimate of the linear map distance between these two closely linked mutants.

There are clearly many similarities between the present results and those obtained in other cases where random spore analysis has been employed.[2, 3, 8, 25, 26] No attempt will be made at this time to interpret all such situations on the basis

of a common mechanism. It is evident, however, that if the present tetrad data have been correctly interpreted as indicating that the majority of pan prototrophs arise by a mechanism other than conventional crossing over, a similar situation may well exist in these cases. Thus it would appear to be extremely difficult, if not impossible, to arrive at a satisfactory interpretation of the mechanism of recombination in such organisms on the basis of random spore isolation data alone.

Summary.—A new group of 37 pantothenic acid-requiring mutants has been obtained from control, X-irradiated, and ultraviolet-irradiated macroconidia of *N. crassa* by means of the filtration-concentration technique. These mutants are all blocked in the same step in pantothenic acid biosynthesis—the conversion of ketovaline to ketopantoic acid. All 37 mutants are located in linkage group VI between the *ad-1* and the *tryp-2* loci and can be considered as alleles at the *pan-2* locus.

Despite their biochemical and genetical similarities, certain mutants can complement to form pantothenic acid–independent heterocaryons. A tetrad analysis has been performed on a cross of two such mutants (*B5* × *B3*) carrying linked markers on either side of the *pan-2* locus. In a total of 939 complete tetrads, exceptional behavior of the pan mutants was noted in 11 asci. On the basis of both heterocaryon and genetic tests, four different types of exceptional asci were obtained having the following isolate ratios: (1) two asci—1 wild type: 1*B3*:1*B5*: 1 double *B5 B3;* (2) four asci—1 wild type:1*B5*:2*B3;* (3) two asci—1 wild type:1*B3*:2*B5;* and (4) three asci—1*B5*:3*B3*. In the two asci of type 1 the adjacent linked markers indicate that the *B3* and *B5* isolates are non-crossover (parental) types, while the wild types and the *B5 B3* doubles are reciprocal single crossover types. This evidence is taken to indicate that the *pan-2* "locus" can be separated into two regions on the basis of genetic (crossover) as well as functional (heterocaryon) tests.

Possible explanations for the three additional types of exceptional tetrads—those with one wild type but no double *B5 B3* or with a 3:1 ratio of mutant types—have been considered. Cytological and genetical evidence argues against an explanation based on heteroploidy. The origin of the three aberrant tetrad types is attributed to some kind of "copy-choice" mechanism which results in irregular segregations for either the *B5*, the *B3*, or both the *B5* and *B3* regions simultaneously. Such "copy-choice" events appear to be correlated with specific patterns of conventional crossing over in the immediate vicinity of the *pan-2* locus.

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*MODIFICATION OF ULTRAVIOLET-INDUCED MUTATION FREQUENCY AND SURVIVAL IN BACTERIA BY POST-IRRADIATION TREATMENT**

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A hypothesis has been advanced that nucleic acid precursors altered *in vivo* by ultraviolet radiation (U.V.) constitute chemical intermediates in U.V.-induced mutation.[1] This theory is based on observations that supplementation of the immediate pre-irradiation growth medium of *Escherichia coli* strain B with certain purines and pyrimidines leads to increases in the mutation frequency subsequently induced by U.V. These studies and those of Witkin[2] indicate that the induction process is related to post-irradiation protein synthesis. It was therefore suggested that the process of U.V.-induced mutation involves post-irradiation synthesis of nucleic acid from radiation-modified precursors; and this process is dependent on *concurrent* protein synthesis.[1]

The experiments reported here were designed to investigate the immediate post-irradiation processes which influence mutation in *E. coli* strain B and the tryptophan-requiring strain of *E. coli* used by Witkin. In addition, considerable information has been accumulated on the post-irradiation conditions influencing survival of this organism following U.V. exposure.

Materials and Methods.—The mutations of *E. coli* strain B studied were those giving aberrant colonial color response on Difco eosin–methylene blue agar (EMB) after 2 days' incubation at 37° C.[3] This particular class of mutants is advantageous for studies in which the surviving fraction is small, since both survival and mutation frequency are determined with the same medium and on the same plates. The basal growth medium was a salts-glucose medium (hereinafter called "*M* medium").[1] *M* medium was supplemented with various metabolites or antimetabo-

lites as indicated in each experiment. All biochemicals used were supplied by the California Foundation for Biochemical Research, Los Angeles, and certified chemically pure. The basic procedures followed for culture growth, radiation exposure, cell treatment, and plating have been previously described.[1] In all experiments reported here the procedure was to synchronize the 16-hour cultures by holding at 6° C. for 1 hour, then to incubate for 50 minutes in minimal medium (or, if so stated, minimal medium with additives) prior to U.V. exposure. The output of the U.V. source used at wave lengths below 2800 A is 96.4 ergs/mm^2/sec at the position of the cells (30 cm. from source). Following irradiation, the cell suspensions were either plated immediately or, in experiments concerned with the post-irradiation events, diluted one in ten into *M* medium containing various supplements. These suspensions were then incubated for varying periods on a reciprocal shaker at the desired temperature (usually 37° C.) and plated as above. All procedures during and following radiation exposure were carried out under yellow light or in the dark, to prevent photoreactivation.

A culture of *E. coli* strain WP2 (a tryptophan-requiring mutant of *E. coli* strain B/r) was kindly furnished by Dr. Evelyn M. Witkin. Induction of reversion to the non-requiring state by U.V. was studied in this strain, using techniques identical with those described for "EMB color" mutation except that *M* medium used in growth was supplemented with 2 mg. DL-tryptophan/100 ml. Plating was onto *M* medium supplemented with 2.5 per cent nutrient broth as described by Witkin.[2] On this medium reverted colonies appear after 3 days' incubation at 37° C. as larger colonies at lower dilutions, while total survivors are counted as smaller colonies at an appropriate higher dilution. *M* medium plus 2.5 per cent nutrient broth provides adequate amino acids for mutation expression of this strain.

Post-irradiation Factors Leading to Decline in Induced Mutation Frequency.—Witkin[2] has suggested that immediate post-irradiation synthesis of protein is required for expression of induced prototrophs with certain auxotrophs of *E. coli* and *Salmonella typhimurium*. The basis for this thesis was that (1) expression of induced prototrophs is directly related to the availability of a complex supply of amino acids during the first hour of post-irradiation incubation; (2) chloramphenicol, an antimetabolite shown to interfere with protein synthesis, interferes with the expression of prototrophs if the cells are treated within the hour following U.V. exposure. We have shown the existence of a similar relation of mutation expression to amino acid supply and inhibition of protein synthesis by chloramphenicol in the case of the "EMB color" mutants.[1] We further demonstrated that the increase in mutation frequency promoted by pre-irradiation incubation in purines and pyrimidines is also dependent on the post-irradiation amino acid supply. Involvement of protein synthesis in processes of mutagenesis has been studied with the "EMB color" mutation system by observing the effects of various conditions limiting to amino acid or protein synthesis on U.V.-induced mutation. The procedure was to retain the cells in limiting medium for periods of time varying from 0 to 90 minutes, then to plate diluted aliquots onto EMB agar medium (which contains an adequate supply of amino acids). Results of a typical experiment, where nitrogen-dependent synthetic activities were limited during the first hour of post-irradiation incubation by holding the cells in *M* medium without the ordinary nitrogen source (ammonium sulfate), are given in Figure 1. Decline in mutation frequency starts imme-

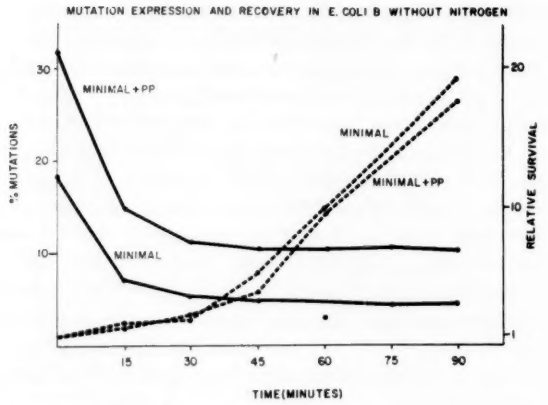

FIG. 1.—Modification of U.V.-induced mutation frequency and survival with incubation in minimal without nitrogen source, ammonium sulfate ("EMB color" mutants of *E. coli* strain B). U.V. exposure was 25 sec. at 30 cm. (2,410 ergs/mm² below 2,800 A). Irradiated cultures were incubated on a reciprocal shaker at 37° C. following exposure. After the indicated incubation time, samples were plated onto EMB agar medium, incubated for 2 days at 37° C., and the mutation frequency and survival determined. The solid line represents mutations; the broken line survival. The cultures were incubated for 50 minutes prior to U.V. exposure in minimal medium or minimal medium plus the purines and pyrimidines (*PP*): adenine, guanine, uracil, and cytosine (1 mg of each/100 ml) as indicated.

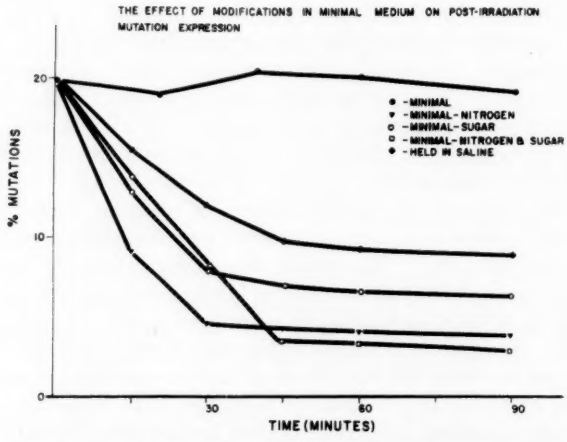

FIG. 2.—The effect of modifications of minimal medium on U.V.-induced mutation frequency ("EMB color" mutants of *E. coli* strain B). Incubation was at 37° C. Cultures were exposed to 25 sec. of U.V. at 30 cm. (2,410 ergs/mm² below 2,800 A) prior to incubation. Following incubation for the indicated time, samples were plated onto EMB agar medium, incubated for 2 days at 37° C. and the mutation frequency determined.

diately and proceeds to a basal level within 30 minutes' incubation at 37° C. The decline rate is similar in cultures incubated in *M* medium prior to irradiation and those which have had their capacity for induced mutation increased by pre-irradiation incubation in the presence of purines and pyrimidines. The residual induced mutation frequency remaining after 30 minutes' incubation without a nitrogen source may be due to amino acids present in the cell at initiation of incubation. Figure 1 also indicates that there is an increase in U.V. survivors when the cells are incubated in the absence of nitrogen. This recovery is not correlated with "mutation-frequency decline," since the latter takes place during the first 30 minutes of incubation, while recovery requires a lag of 30–45 minutes before initiation.

A similar decline in mutation frequency is demonstrated in Figure 2 with post-irradiation incubation in the absence of an energy source (glucose). In this case the rate of decline is less rapid than that observed in the absence of nitrogen. In the absence of both nitrogen and glucose the rate of decline remains similar to that observed with absence of glucose alone. This suggests the possibility that absence of an energy source is limiting to processes responsible for the observed decline in mutation frequency and suggests that these processes may be enzymatic. Other components of *M* medium are also needed for this decline in mutation frequency, since in saline the decline is limited as to both rate and final level attained.

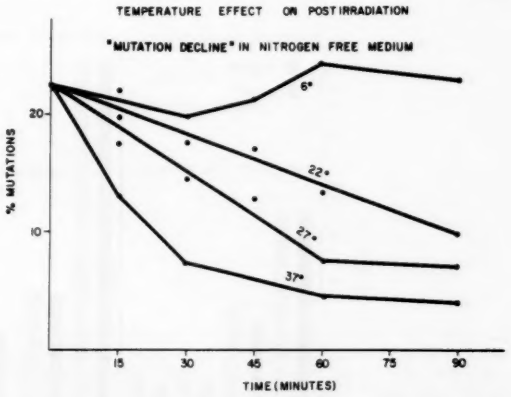

FIG. 3.—The effect of post-irradiation incubation temperature (° C.) on U.V.-induced mutation frequency in nitrogen-free medium ("EMB color" mutants of *E. coli* strain B). After incubation for the indicated time, samples were plated onto EMB agar medium, incubated for 2 days at 37° C. and the mutation frequency determined. The U.V. exposure was 25 sec. at 30 cm. (2,410 ergs/mm² below 2,800 A).

Similar considerations apply to increases in survival which are observed. Recovery is limited both in time of initiation and in rate by the absence of glucose.

Further experiments carried out to investigate the nature of the mutation-decline process show that decline is prevented for 90 minutes by holding the irradiated cells in an ice bath; but when the cells are then warmed to 37° C., the typical decline is observed. Figure 3 shows the rate of "mutation-frequency decline" observed at several post-irradiation temperatures. The rate of decline is retarded by lowering the temperature from 37° C. It has been established from such experiments that the "mutation-frequency-decline" process has a Q_{10} of approximately 2 between the range of 37° and 22° C., suggesting a typical enzymatic reaction. It is possible that, in the absence of amino acids or an available nitrogen source, an enzymatic process occurs which removes the potential mutation before it is "fixed" into the genetic structure of the cell. If this is the case, the role of amino acids in mu-

tation induction could be stabilization of the mutation (or mutagen), removing it from enzyme action until the process of "mutation fixation" is completed.

In view of the foregoing, it appeared important to determine the role of chloramphenicol in interfering with mutation induction. Interference with "mutation stabilization" would indicate that chloramphenicol acts to prevent this process and consequently allows the enzymatic process causing "mutation-frequency decline" to occur in a manner similar to that effected by limitation of amino acids or available nitrogen. Interference with "mutation fixation" would point significantly to involvement of protein synthesis in the final mutation process. Figure 4 shows that there is no significant difference in the "mutation-frequency decline" observed when comparing chloramphenicol treatment with nitrogen deprivation. Therefore, cloramphenicol may act by preventing "mutation stabilization" by amino acid–dependent processes, thus allowing enzymatic "mutation-frequency decline" to proceed.

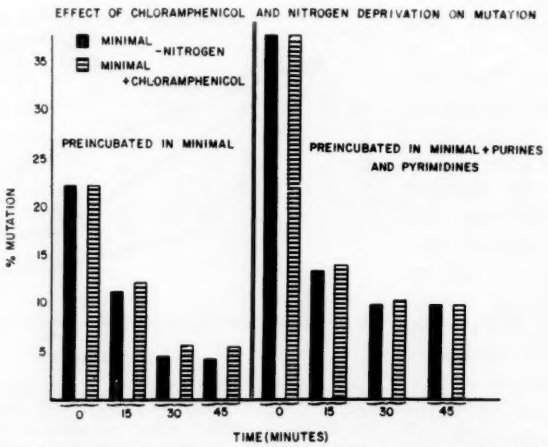

FIG. 4.—Comparison of the effects of nitrogen deprivation and chloramphenicol on U.V.-induced mutation frequency ("EMB color" mutation of *E. coli* strain B). The U.V. dose was 25 sec. at 30 cm. (2,410 ergs/mm² below 2,800 A). Following irradiation, the cultures were incubated at 37° C. in minimal medium minus ammonium sulfate or in minimal medium to which chloramphenicol (20 μg/ml) was added. Following incubation for the indicated time, aliquots were withdrawn, diluted, and appropriate dilutions plated onto EMB agar medium. These plates were incubated for 2 days at 37° C. and the mutation frequency determined. One culture was incubated prior to U.V. irradiation in minimal medium for 50 minutes and a second culture for the same time in minimal plus adenine, guanine, uracil, and cytosine (1 mg of each/100 ml) (*PP*).

Time Course of Mutation Fixation.—In view of the above results, the time course of the "mutation-fixation" process following U.V. exposure can be established by utilizing enzymatic removal of potential mutations by nitrogen deprivation or chloramphenicol treatment. Those mutations not affected by this treatment have presumably been "fixed" into the genetic structure of the cell—or are at least past the point of attack of the enzymatic process leading to "mutation-frequency de-

cline" prior to introduction of the drug or complete nitrogen deprivation. This, in effect, is the process which the data of Witkin[2] on auxotroph reversion may be presumed to have measured. She showed that after a period of 60–90 minutes none of the induced mutations were subject to "reversal" by chloramphenicol treatment. In the case of the "EMB color" mutations a similar time course for "mutation fixation" occurs (Fig. 5). No significant fixation occurs for 30 minutes. Following this, a gradual removal of potential mutations from "chloramphenicol challenge" takes place and is essentially complete at 90 minutes' incubation.

The Effect of Nucleoside Antagonists.—It was not possible, on the basis of the accumulated evidence, to determine whether RNA or DNA synthesis (or both) is directly involved in the increase in mutation frequency occurring with pre-irradiation incubation with purines and pyrimidines.[1] The fact that uracil is effective and not thymine in this effect suggests that RNA synthesis is directly involved. However, the possibility of uracil conversion to thymine remains. The label of C-14 uracil is incorporated both into RNA and DNA by *E. coli*.[4] Furthermore, utilization of exogenous thymine by *E. coli* strain B in DNA formation is slight.[4] For these reasons the singularity of uracil in the purine and pyrimidine effect does not serve as any indication that RNA synthesis, as opposed to DNA synthesis, is immediately involved in the mutation-fixation processes.

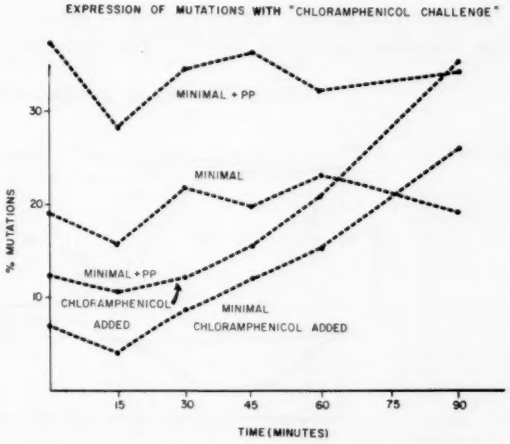

FIG. 5.—Effect on U.V.-induced mutation frequency of chloramphenicol added after various post-irradiation incubation intervals ("EMB color" mutation of *E. coli* strain B). U.V. exposure was 25 sec. at 30 cm. (2,410 ergs/mm² below 2,800 A). Cultures were incubated following irradiation in minimal medium supplemented with 2 mg/ml casein hydrolysate. After the indicated intervals, chloramphenicol (20 μg/ml) was added and the cultures incubated for an additional 45 minutes. Two sets were run: in one set (labeled *minimal*) the cells were incubated for 50 minutes, prior to U.V. exposure, in minimal medium; in the second (labeled *minimal + PP*) the cells were incubated for 50 minutes, prior to U.V. exposure, in minimal medium plus adenine, guanine, uracil, and cytosine (1 mg of each/100 ml). The upper curves for each set represent the mutation frequency of the culture without chloramphenicol treatment. The lower curves for each set (labeled *chloramphenicol added*) represent the mutation frequency of the culture incubated for the indicated time before chloramphenicol was added. These cultures were then incubated an additional 45 minutes. Following incubation, the cultures were plated on EMB agar medium and incubated at 37° C. for 2 days.

Studies have demonstrated that the ribosides (adenosine or guanosine, cytidine, and uridine) are uniquely effective among the various nucleosides tested in increasing the U.V.-induced mutation frequency. The deoxyribosides are without any significant effect. However, objections to the assumption that RNA synthesis is directly involved also apply here, since the ribosides could be converted to deoxyribosides in DNA synthesis. Resolution of this question was approached ex-

perimentally by addition of antagonists of uridine or thymidine to the immediate post-irradiation growth medium. The analogue, 5-hydroxydeoxyuridine, which antagonizes thymidine incorporation into DNA, has little effect on mutation frequency following U.V. exposure. However, this cannot be considered as ruling out participation of DNA synthesis in the immediate "mutation-fixation" process. The ability of 5-hydroxydeoxyuridine effectively to block DNA synthesis in *E. coli* strain B has not been established. The question of involvement of DNA synthesis in the "mutation-fixation" process is under extensive investigation in our laboratory at the present time.

A uridine antagonist, 5-hydroxyuridine, shows a marked effect on post-irradiation mutation expression (Fig. 6). The analogue, 5-hydroxyuridine, is highly ef-

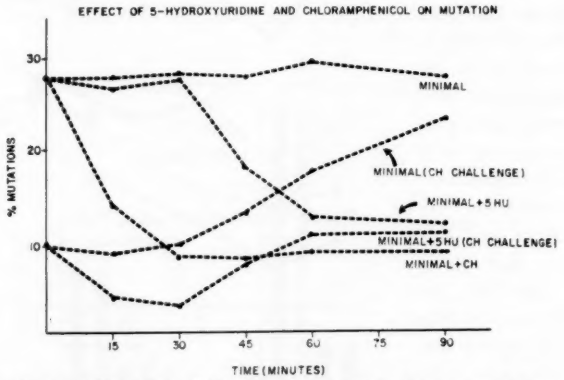

FIG. 6.—The effect of U.V.-induced mutation frequency of incubation with 5-hydroxyuridine and subsequent chloramphenicol treatment at various post-irradiation incubation intervals ("EMB color" mutants of *E. coli* strain B). U.V. exposure was 25 sec. at 30 cm. (2,410 ergs/mm² below 2,800 A). The bacteria were incubated following irradiation in minimal medium (supplemented with 2 mg/ml of casein hydrolysate) with and without 5-hydroxyuridine (5HU) (5 mg/100 ml). After various times an aliquot was plated immediately (labeled *minimal* or *minimal + 5HU*). At these same times 10 μg/ml chloramphenicol as added to an identical aliquot which was incubated for an additional 45 minutes and plated (*CH challenge*). The curve labeled *minimal + CH* represents a culture to which chloramphenicol was added at the beginning of incubation. All plating was onto EMB agar and incubation was for 2 days at 37° C.

fective in reducing protein and RNA syntheses, as well as in blocking adaptive enzyme formation in *E. coli*.[5] However, treatment of irradiated cells with this antagonist does not lead to "mutation-frequency decline" during the first 30 minutes following exposure, as does chloramphenicol treatment or nitrogen deprivation. Full capacity for subsequent induced mutation expression is retained during this time, although there is little protein synthesis. A marked decline is observed between 30 and 60 minutes' incubation. The effect of "chloramphenicol challenge" on mutation expression during this treatment is instructive. Figure 6 indicates that the period of "mutation fixation" (demonstrated by the decreasing effectiveness of chloramphenicol in reducing mutation frequency) corresponds to the pe-

riod of "mutation-frequency decline" promoted by the uridine antagonist and also shows that "mutation fixation" is limited by "mutation-frequency decline." Conversely, "mutation-frequency decline" is limited by "mutation fixation." Further results have shown that "mutation-frequency decline" in the presence of 5-hydroxyuridine may be prevented by addition of uridine, while thymidine has no effect (for effects of post-irradiation incubation in uridine alone see a later section). Similar results have been obtained with cells which have had their capacity for mutation increased by pre-irradiation incubation in purines and pyrimidines. This evidence, taken together, supports the possibility that ribonucleic acid synthesis is directly involved in the mutation-induction process.

Reversion in E. coli Strain WP2.—Experiments were carried out to determine whether or not comparable results could be obtained in the case of U.V.-induced reversion of the tryptophan requirement of *E. coli* strain WP2. Table 1 indicates

TABLE 1

EFFECT OF POST-IRRADIATION TREATMENT FOR VARIOUS INTERVALS ON YIELD OF INDUCED PHOTOTROPHS IN *E. coli* STRAIN WP2 (TRYPTOPHAN REQ.)

Post-Irradiation Incubation Medium*	Incubation Time (Minutes)	Surviving Organisms Plated (× 10^9)	Induced Prototrophs Plated	Mutants per 10^8 Surviving Organisms
	0	100	5,616	56.2
M + T	20	93	4,533	48.7
M + T + CH		107	8,066	75.4
M − N		120	1,120	9.3
M + T + CH + Chl		75	670	8.9
M + T + CH + 5HU		88	4,333	49.2
M + T	40	108	2,466	22.8
M + T + CH		121	7,333	60.6
M − N		90	926	10.3
M + T + CH + Chl		56	450	8.0
M + T + CH + 5HU		94	2,533	26.9
M + T	60	99	1,586	16.0
M + T + CH		57	5,633	98.8
M − N		112	586	5.2
M + N + CH + Chl		68	323	4.7
M + T + CH + 5HU		98	900	9.2
M + T	80	108	603	5.6
M + T + CH		88	3,866	43.9
M − N		71	570	8.0
M + T + CH + Chl		33	350	10.6
M + T + CH + 5HU		88	360	4.1

*M = minimal medium; T = DL-trypophan, 2 mg./100 ml; CH = casein hydrolysate, 2 mg/ml; Chl = chloramphenicol, 20 μg/ml; 5HU = 5-hydroxyuridine, 5 mg/100 ml; −N = ammonium sulfate deleted. U.V. exposure was for 15 sec. at 30 cm. (1,446 ergs/mm² below 2800 A). Approximately 10 per cent of the irradiated organisms survived this dose. Post-irradiation incubation was at 37° C.

that results comparable to those obtained with the "EMB color" mutation system are obtained with *E. coli* strain WP2. Both nitrogen deprivation and chloramphenicol treatment result in marked "mutation-frequency decline," which is complete to the basal level within 20 minutes. Furthermore, the presence of 5-hydroxyuridine in the post-irradiation growth medium results in a slower, but marked, decline in induced mutation frequency. Pre-irradiation growth medium supplementation with the RNA purines and pyrimidines results in increases in mutation frequency comparable to those observed with "EMB color" mutation in *E. coli* strain B.[1] It is therefore probable that the basic mechanisms involved in induction of "EMB color" mutation and reversion of tryptophan requirement of *E. coli* strain WP2 are identical in nature. In view of these results the mechanisms involved may have general application in U.V.-induced mutation.

Comparison of the rates of "mutation-frequency decline" obtained with *E. coli* strain WP2 by us and by Witkin is instructive. Witkin found that 60–90 minutes' post-irradiation treatment with chloramphenicol was required to reduce the yield of induced prototrophs obtained from irradiated *E. coli* strain WP2 to a basal level unaffected by further chloramphenicol treatment. We find, on the other hand, that an incubation period of less than 20 minutes is sufficient to reduce the yield of induced prototrophs to the basal level. While various differences in technique may enter in, the primary difference between our experiments and those of Witkin which may account for this discrepancy is in the nature of the pre-irradiation culture media. Witkin grew her cultures in nutrient broth rich in amino acids and other organic material. Our pre-irradiation culture medium was a basal salts–glucose medium supplemented with tryptophan. It may be that the discrepancy in results is attributable to differences in intracellular metabolites or synthetic capacities of the cultures at the time of irradiation resulting from the different pre-irradiation culture media.

The fact that "mutation-frequency decline" occurs within a short period with both the "EMB color" mutations and the tryptophan reversion in strain WP2 is highly significant. It is probable that the decline observed is not associated with major cellular macromolecular syntheses or degradations of protein or nucleic acid. No change in the net quantities of these constituents present in the cell are observed in the short period required for "mutation-frequency decline." Furthermore, it is evident that "mutation stabilization" due to the presence of amino acids does not involve the synthesis of a *stable* macromolecule. Mutations may be stabilized for a period of 15–30 minutes in the presence of amino acids; yet essentially complete "mutation-frequency decline" may be observed on the withdrawal of amino acids or chloroamphenicol treatment after this period (see Fig. 5). It seems probable that the potential mutation in the initial stage of the process of U.V.-induced mutation is not associated with a biologically specific macromolecule but rather with a smaller molecule immediately subject to enzyme action. In view of our results,[1] the purine or pyrimidine nucleotides or other nucleic acid precursors appear likely candidates for this role.

The Effect of Post-irradiation Supplementation with Purine and Pyrimidine Ribosides on Mutation Expression.—It has been demonstrated that, while purines and pyrimidines (adenine or guanine, uracil, cytosine) show an effect in increasing mutation frequency only after a considerable lag (15–25 minutes) when supplied to *E. coli* strain B prior to radiation exposure, the corresponding ribosides effect an almost immediate increase in mutation response to ultraviolet light.[1] It was suggested that the nucleic acid precursors must be exposed to radiation for this effect, since immediate post-irradiation addition of purines and pyrimidines to irradiated cells has no effect and, in fact, in some experiments decreased the induced mutation frequency.

This experimentation has been extended by studying the effect of the ribosides on induced mutation frequency when they are added immediately following radiation exposure. It has been established that purine and pyrimidine ribosides (either singly or in combination) supplied to incubating cells immediately after radiation exposure will not increase the induced mutation frequency. This is true for radiation-exposed cultures held either in minimal medium (with ammonium sulfate sup-

plied as the sole nitrogen source) or in minimal medium supplemented with a complex source of amino acids (casein hydrolyzate). This further supports the hypothesis that nucleic acid precursors must be present in the cell *during* radiation exposure and may constitute chemical intermediates in mutagenesis after modification by ultraviolet.

Adenosine and guanosine do not appear to influence mutation frequency, but either cytidine or uridine produces a progressive decrease with incubation (Fig. 7). This would be expected if cytidine and uridine supplied exogenously—or their immediate metabolic products—actually compete with corresponding endogenous radiation-modified precursors in RNA synthesis to reduce induced mutation.

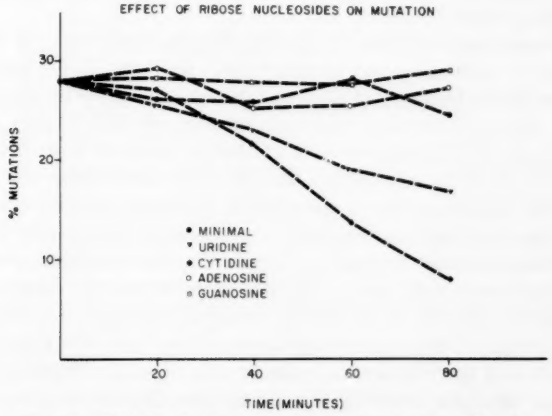

FIG. 7.—The effect of post-irradiation incubation with ribose nucleosides on U.V.-induced mutation frequency ("EMB color" mutation of *E. coli* strain B). U.V. exposure was 25 sec. at 30 cm. (2,410 ergs/mm² below 2,800 A). Incubation was in minimal medium plus casein hydrolysate (2 mg/ml), to which the indicated nucleoside (1 mg/100 ml) was added. At the indicated periods of incubation, aliquots were withdrawn, appropriately diluted, and plated onto EMB agar medium. These plates were incubated for 2 days at 37° C. and the mutation frequency determined.

Discussion.—Based on the accumulated evidence, the following may be stated as an experimental hypothesis for U.V.-induced mutation in bacteria. More investigation at the biochemical and genetic levels with several different systems is necessary to establish the validity of the hypothesis and to determine whether or not it is a general mechanism for U.V.-induced mutation.

1. The principal mutagenic effect of ultraviolet light is to modify ribonucleic acid precursors present in the cell at the time of radiation exposure.

2. These modified precursors are stabilized in the cell by an enzymatic process dependent on a source of amino acids ("mutation stabilization"). If the precursors are not stabilized, they are rapidly removed (or lose their capacity to effect mutation) by a second enzymatic process ("mutation-frequency decline").

3. Mutation is exerted through the process of incorporation of the modified nucleic acid precursors with RNA synthesis, resulting in modification of the RNA

("mutation fixation"). The evidence suggests that concurrent synthesis of ribonucleic acid and protein is immediately involved.

4. The nature of the link, if any, between modification of the RNA and modification of the cellular DNA (the gene), though suggested by these results, is unknown. It may be that RNA and protein syntheses are involved in replication of DNA, and changes in RNA specificity result in corresponding changes in genic DNA. Adequate evidence does not exist at present that the "EMB color" mutations studied involve a DNA-containing hereditary system. However, the transducible substrain of *Salmonella typimurium* (try 3) used in the experiments of Witkin[2] showing a similar dependence of mutation expression on the post-irradiation supply of amino acids evidently has the tryptophan-requiring gene in its DNA-containing hereditary system.

No concrete information exists concerning the chemical nature of the suggested mutagenic nucleic acid precursor at this time; however, recent studies by Sinsheimer[6] on the photochemistry of ribo- and deoxyribonucleotides are interesting in this respect. His work demonstrates that exposure of uridylic acid and cytidylic acid to U.V. produces relatively unstable irradiation products from these pyrimidine nucleotides. None of the other ribo- and deoxyribonucleotides demonstrate this property. Although the nature of the unstable substance produced by U.V. is unknown, it is suggested that it is the result of addition of water across the 5,6 double bond.[7] Evidence exists for this particularly in the case of uridylic acid.[7] No evidence exists that these radiation products possess mutagenic capacities, but this possibility must be considered in view of the results presented here. Decay, by loss of water during or following incorporation of these unstable radiation products into the nucleic acid macromolecule, could produce configurational changes of the macromolecular structure, resulting in a change in its hereditary potentiality. The possibility exists that U.V. modification of a pyrimidine-building unit of the nucleoprotein molecule synthesized prior to exposure could produce a change in configuration resulting in mutation.[6, 7] However, the nucleoprotein macromolecule may be a stable structure resistant to this type of U.V. modification. These considerations imply that nucleic acid replication involves configurational instabilities subject to the disrupting effect of certain modified building blocks. Further insight into the phenomena of mutation must await increased understanding of the nature of nucleic acid replication and protein synthesis and the macromolecular configurational aspects of genetic specificity.

The nature of the process of "mutatation stabilization" suggested by our data has not been determined. However, it is evident that the principal participants in this process are nucleic acid precursors and protein precursors. Recent studies implicate nucleotide–amino acid complexes as precursors to protein formation.[8] It has been suggested that such a complex could also serve as an immediate precursor to nucleic acid formation.[8] It may be that the nucleic acid precursor–amino acid interaction involved in stabilization of pyrimidine "mutagens" from enzymatic attack ("mutation-frequency decline") is formation of the nucleotide–amino acid complex suggested above. However, it is evident that something more than the formation of an amino acid–nucleotide complex is involved in "mutatation stabilization," since, from available evidence,[8] chloramphenicol does not interfere with the formation of the nucleotide–amino acid complex and since we have shown that it allows

"mutation-frequency decline" to take place. Rather, a necessary subsequent process involved in "mutation stabilization" and interfered with by chloramphenicol is suggested by our results.

Further speculation as to the nature of the biochemical processes involved in "mutation-frequency decline," "mutation stabilization," and "mutation fixation" is pointless at this time. However, the results presented suggest that U.V.-induced genetic change or mutation is exerted through the basic processes of genic replication. These processes apparently involve the synthesis of DNA, RNA, and protein. Further study of the nature of mutagenesis may yield information relevant not only to the mechanism of genetic change but also to the basic biochemical interrelations involved in genic replication and possibly gene action in enzyme formation.

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*THE INITIATOR CELL FOR SLIME MOLD AGGREGATION**

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INTRODUCTION

The cellular slime molds, Order Acrasiales, belie their Protistal status by cooperating to produce organized multicellular fruiting bodies. These exhibit a significant degree of cellular differentiation and require for their construction extensive morphogenetic movements. The process begins when the population of myxamoebae has reached the stationary phase and need not involve further cell division.[1] Cellular endogenous reserves amassed during the lag and log phases are sufficient to fuel the process, and no exogenous materials need be supplied.[2, 3] The

first stage—aggregation—brings the heretofore independent myxamoebae together at central collecting points. The cells, excited by a chemotactic stimulus,[2] elongate and orient radially and move toward the aggregative centers in ramified streams (Fig. 1). The resulting conical masses of cells become organized ultimately into fruiting bodies.

Washed myxamoebae have been dispensed on washed agar at controlled population densities and allowed to aggregate.[3] At the optimal density, the particular value being a function of species and strain, the number of aggregative centers was directly proportional to the number of cells present. The particular value of the

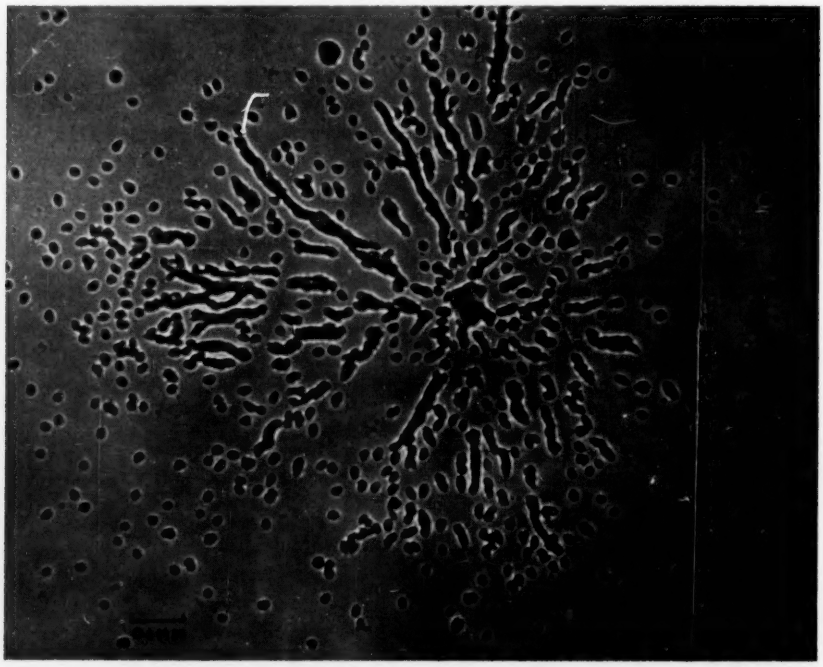

FIG. 1.—An early aggregate. This is from a series of time-lapse photomicrographs. The central clump contained an I-cell around which neighboring cells had congregated. Subsequently, concentrically placed clumps appeared and elongated to become streams. Later photographs show much more drastic cell elongation and excitation over a greater area.

ratio of centers:cells also was found to be species- and strain-specific. Further, only a minor proportion of the cells proved to be capable of initiating the formation of these aggregates. Among small replicate population samples at high density, aggregative centers were distributed statistically as a single event (i.e., in accord with the Poisson distribution). These and subsidiary data[4-6] compelled us to conclude that the formation of an aggregate requires the presence of a specially endowed cell, termed the "initiator," which induces its neighbors to begin aggregation.

It was recognized that visual identification of the initiator cell could make it

possible to answer directly questions relating to the process of cellular differentiation implied herein and thereby to elucidate the genetic basis of the difference between the initiator and responder cells and to examine the specific interactions between these two types which lead to the onset of aggregation. The present communication is concerned with an attempt to supply this identification, apparently a successful one.

The following criteria were employed to examine the validity of our choice:

1. The cell must be accurately distinguishable by inspection prior to the onset of aggregation.
2. The cell must be present in a proportion consistent with the distribution of aggregative centers.
3. A high correlation must exist between the positions of the supposed initiator cells and of the aggregates that subsequently appear.
4. In small population samples which display a Poisson distribution of aggregative centers, the incidence of the supposed initiator cells and of centers must be perfectly correlated.
5. Removal of the supposed initiator cell prior to the completion of its task must prevent the occurrence of a center.
6. Movement of the supposed initiator cells by micromanipulation must result in the induction of a center at the new position.

METHODS

Detailed descriptions of culture methods have been given elsewhere.[3-5] *Dictyostelium discoideum* Raper, strain NC-4 wild type, was grown on SM agar in association with *Aerobacter aerogenes*. After 40 hours' incubation at 22° C., the cells had reached the stationary phase. The myxamoebae were harvested and washed free of occluded bacteria by differential centrifugation[3] and were counted in a Levy hemocytometer. Appropriate dilutions in salt solution[2] were dispensed in measured drops on washed agar. The plates were allowed to stand with covers ajar until the excess fluid was absorbed, leaving the myxamoebae distributed homogeneously within the confines of the drop. Such cells begin aggregation within 12–14 hours and without detectable increase in either direct or viable count.

The washed agar was prepared by suspending 25 gm. Difco bacto-agar in 1 liter distilled water and filtering through a table-top Buchner funnel. This was repeated five times, and the agar was melted in 1 liter of water. For special purposes to be described, 12.5 gm. agar, washed with 9 liters and melted in 500 cc. water, was employed. The use of washed agar not only prevents the growth of the myxamoebae but also restricts their movements largely to within the confines of the drop of salt solution in which they were dispensed. Very probably the divalent cations present, particularly calcium, act as a trap to prevent spreading, since these ions are required for motility.[1]

RESULTS

A. *Recognition of the I-Cells.*—Washed Myxamoebae were dispensed on 6× washed agar in 0.01-ml. drops at a population density of 200 cells/mm^2. The plate covers were left ajar for 20 minutes to permit absorption of the excess fluid. After 4 hours' incubation, the cells were examined microscopically at 100× and 440× magnification.

Two cell types were recognized. These are shown in Figure 2. The majority of the cells were small, with a mean diameter of 11 $\mu \pm 16$ per cent, in agreement with previous estimates,[6, 7] hemispherical in silhouette, and round or elliptical in outline. They rarely extended pseudopodia and moved very sluggishly. In contrast, a few cells were much larger, with a diameter three to ten times that of the first group and were extremely flat in silhouette, causing them to acquire a bluish tint without highlights at certain angles of illumination. Extensive, almost explosive, lobose pseudopodia emerged from these cells constantly. In consequence, they moved about at a great rate but in random directions. They were also more heavily granulated than the majority type and showed a much higher rate of protoplasmic streaming and of contractile vacuole formation and disappearance. The resting nuclei, recognized under phase contrast as being the same structures which stain

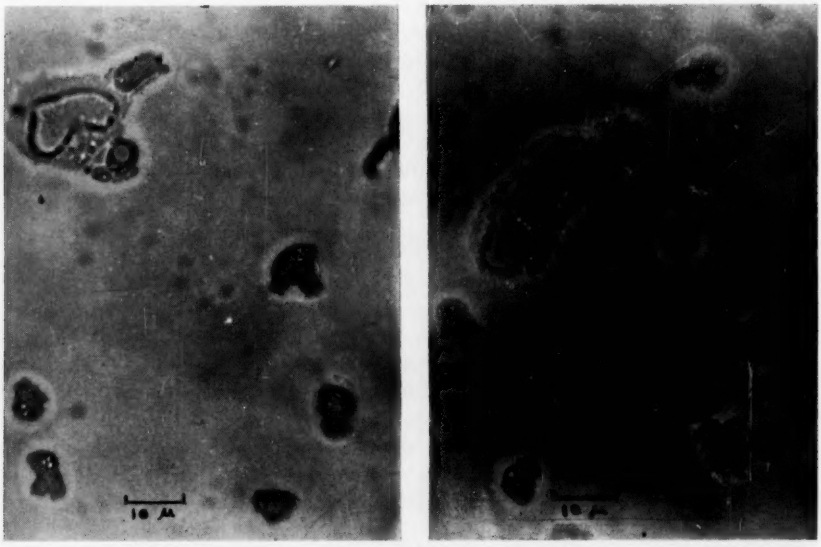

FIG. 2.—Two I-cells shown with neighboring responder cells. Note the extreme flatness of the former, as indicated by their greater transparency. The tendency to contact and encircle responder cells is very marked but has not been seen to result in engulfment.

with iron haematoxylin,[1] were much larger than those of the majority type in approximate correspondence with the total cell size difference. The individuals of this minority type were termed *I-cells.*

Experiments involving laboratory personnel have indicated that the I-cells are not obvious to the inexperienced eye but, once seen, are recognized thereafter with ease and high precision. Preliminary identification by size and flatness can be accomplished under low magnification. Confirmatory recognition of the explosive pseudopodia, high rate of motion, and granulation requires 440× magnification. Detailed cytological data will be published elsewhere.

B. *The Proportion of I-Cells in the Population.*—Washed myxamoebae were dispensed as before on 6× washed agar at a density of 200 cells/mm^2. Squares of 1 mm^2 in area were scored on the agar, using two pieces of razor blade mounted in

parallel at a distance of 1 mm. Total cell and I-cell counts were made within the confines of these squares. The data are summarized in Table 1 for cells incubated for periods of 3.5, 5, and 10 hours. The first two estimates were obtained from single experiments; the last from four independent determinations which showed a standard deviation of 5.5 per cent. As seen, the I-cell count remained constant at 1:1,940 during the preaggregative period.

TABLE 1

POPULATIONAL DISTRIBUTION OF I-CELLS

Incubation Period Hours*	No. Cells Examined	No. I-Cells	Ratio of I-Cells to Total Cells
3.5	17,160	9	1:1910
5	18,720	9	1:2090
10	114,300	59	1:1940 ± 107 σ

* The incubation period was measured from the time at which the cells were dispensed on washed agar.

Companion drops on the washed agar were left unscored, and counts of aggregative centers were made from these. Experiments carried out over the last 6 years have shown that, at a density of 200 cells/mm^2, optimal for NC-4 wild type, the ratio of centers formed to cells present is 1:2,200.[3] In the experiments performed here, a ratio of 1:2,420 was obtained. Thus the proportion of I-cells in the population can be seen to exceed by about 20 per cent the distribution of aggregative centers.

C. *Correlation between the Positions of I-Cells and of Centers.*—These experiments are summarized in Table 2. Washed cells were dispensed as before at 200 cells/mm^2. After 8–10 hours, low-power fields containing I-cells were chosen and their positions fixed by taping each plate to a microscope stage. The radius of these fields was 750 μ. Thereafter, the plates were uncovered at intervals, so that drawings could be made designating the positions of each I-cell and its immediate neighbors with

TABLE 2

CORRELATION BETWEEN POSITIONS OF I-CELLS AND OF AGGREGATIVE CENTERS

Category*	Result	No. Fields	Per Cent of Total
A	Center formed at I-cell	18	37.5
	Center formed nearby	19	39.5
	Total	37	77.0
	No center in field	11	23.0
B	Center formed at I-cell	11	50.0
	Center formed nearby	6	27.3
	Total	17	77.3
	No center in field	5	22.7
C	Center in field	7	25.0
	No center in field	21	75.0

* A = predictive success by choice of fields containing I-cells; B = predictive success in randomly chosen fields containing I-cells; C = predictive success in randomly chosen fields not examined for the presence of I-cells.

respect to reference particles of debris. The I-cells moved fairly rapidly but randomly and always remained within the original field, generally within a radius of a few hundred micra. A total of 48 fields was examined in this fashion (category A in Table 2). In 37 fields (77 per cent), centers formed at or near the I-cells. In 11 fields (23 per cent), no centers appeared. As controls, fields were chosen at ran-

dom without inspection and fixed by taping the plates to microscope stages. Of 28 fields (category C in Table 2), centers occurred in 7 (25 per cent), no centers in 21 (75 per cent). Thus the chance of including a center in a field when an I-cell was present was threefold greater than in randomly selected fields.

A detailed examination of the experiment shows that the correlation was even more significant than is indicated above. When centers appeared in the fields containing I-cells, the first signs of aggregation occurred after 11.5–12.5 hours' incubation. Almost invariably, a few myxamoebae nearest the I-cell moved toward it and nestled against it. Within a few minutes large cell clumps formed concentrically about the I-cell at distances between 50 and 200 μ. Clumps larger than two to three cells never formed prior to this time. In 18 of the 37 cases, cells continued to congregate around the I-cell until it could no longer be distinguished within the mass. Outlying cells then elongated and became radially oriented, followed by similar orientation by the cells within the outlying clumps. All began to move toward the I-cell clump, so that by 12.5–13.5 hours the streams were well established. Thus in 18 cases (37.5 per cent), the center occurred at the exact position of the I-cell. Because the ratio of centers: cells at optimal density for *D. discoideum* is 1:2,200, the random chance of predicting that a center would coincide with the position of a single cell is 0.045 per cent.

In the remaining 19 cases the center formed nearby. The sequence was the same, except that, after the concentric clumps had formed, the cells moved not toward the I-cell but toward a nearby clump, invariably very large and practically always less than 200 μ distant from the I-cell. In half of these cases the I-cell itself entered the favored clump. In the other half the I-cell remained alone outside of the aggregate.

Of the 11 negative cases in category A, the cells of 3 of these were not drawn to any center. In 8 cases the cells were attracted to a center outside the field and streamed toward it. In 6 of these, the I-cell remained unattracted.

It was realized that our choice of fields might be dictated by a subjective impression unconnected with the presence of an I-cell. Accordingly, a set of fields was chosen without inspection, and the plates were taped to microscope stages. Then those fields containing I-cells were retained for study (category B in Table 2). Of 22 fields, 17 produced a center at or near the I-cell for a predictive success of 77.3 per cent, again threefold greater than background. In the 17 successful cases, the center coincided with the position of the I-cell in 11 (50 per cent of the total), to be compared with the calculated background of 0.045 per cent.

Time-lapse camera lucida drawings and photomicrographs of these aggregations will be published elsewhere.

D. *Correlated Incidence of I-Cells and Aggregates in Small-Population Samples.*—As mentioned previously, the supposition that randomly distributed initiator cells were responsible for the formation of aggregates was predicated in part upon the fact that small replicate populations of myxamoebae displayed a Poisson distribution of aggregates. This system now provided an opportunity to demonstrate the extent to which, in such samples, the distribution of centers was correlated with the incidence of I-cells.

Replicate samples of 500 washed myxamoebae were dispensed on 6× washed agar, using a machined platinum loop (volume delivered, 1.1×10^{-4} ml. $\pm$ 0.1×10^{-4} σ). After 8 hours, the population density was approximately 300 cells/mm^2.

At this time, the drops were surveyed to determine which ones contained I-cells. At 16 hours the drops were examined for the presence of aggregative centers and again at 30 hours to make sure that no additional ones had appeared in the interim. As shown in Table 3, centers appeared in 18 of the 32 drops with I-cells and in only 2 of the 67 drops without I-cells. The probability that this distribution could be equaled or exceeded by chance is less than 0.001 by chi-square test. The over-all ratio of centers to cells was 1:2,170, in agreement with previous estimates.[3]

TABLE 3

CORRELATION BETWEEN INCIDENCE OF I-CELLS AND AGGREGATION CENTER IN SMALL-POPULATION SAMPLES

SAMPLES	EXPERIMENTS A	B	C	TOTAL
With I-cells				
No. with centers	7	8	3	18
No. without centers	5	6	3	14
Without I-cells				
No. with centers	1	1	0	2
No. without centers	18	28	19	65
Total = 99			$\chi^2 = 37.27$	
No. with centers = 20			d.f. = 3	
Ratio centers:cells = 1:2,170*			$p < 0.001$	

* Calculated from the P_0 term of the Poisson series.

That only 60 per cent of the samples with I-cells aggregated is probably explained by our zealousness in choosing I-cells, which injected a subjective bias into the experiment. At densities appreciably greater than 200 cells/mm^2, recognition of I-cells is complicated by the fact that they are often in contact with neighboring cells and cannot attain their full diameter or display their high rate of motility. Eventually, they become recognizable by sustained microscopic observation, since, upon disengaging from their neighbors, the I-cells can be readily distinguished. The element of doubt lay with cells which were slightly larger than the average and likewise in contact with neighbors. Sustained observation produced no evidence that these were I-cells, but—and herein lies the subjective bias—they were recorded as such. Accordingly, the estimate of I-cell number was significantly higher than those shown in Table 1. The doubtful cases represented about 20 per cent of the 32 samples and are sufficient to have accounted for the low figure observed.

The validity of this explanation has been substantiated in three subsequent experiments, performed for another purpose, which required that we make unqualified choices of small-population samples with and without I-cells. Twenty-six samples were so chosen, 13 with I-cells and 13 without. Of the former, 100 per cent aggregated, none of the latter. Thus, in a limited series where one need not make a decision on every sample but can merely present one's unquestionable choices for test, a perfect correlation between the incidence of I-cells and aggregative centers can be obtained.

That 2 of the 67 drops without I-cells aggregated could be taken as evidence that an alternate mechanism of aggregation not involving I-cells exists. If so, it obviously can account for only a very minor proportion of aggregate formation under the conditions employed. It is equally possible that two I-cells went unrecognized, particularly in view of the difficulty of their identification at this population density.

E. *Effect of Prior Removal of I-Cells on Center Formation.*—The experiments to

be described, which are summarized in Table 4, indicate, first, that removal of I-cells can wipe out center formation completely and, second, that the I-cell completes its task within an amazingly short time after deposition on washed agar.

The first experiments toward this end consisted of dispensing replicate populations of 4,000 myxamoebae on washed agar at a density of 200 cells/mm^2. After 8 hours, all detectable I-cells were removed by micromanipulation (see next section for experimental details). Subsequent counts of aggregative centers revealed no difference between experimentals and controls in which the I-cells had been retained. Realization that the I-cells might already have fulfilled their function by 8 hours dictated removal at an earlier time, but manipulation at 4 hours and then at 1 hour still failed to result in any difference between experimentals and controls in capacity to produce aggregates. Finally, removal at 20 minutes provided a 33 per cent decrease in center formation.

The definitive experiments, the results of which are given in Table 4, were performed as follows. Myxamoebae were exhaustively washed by seven differential centrifugations,[3] suspended in salt solution, and maintained in an ice bath prior to

TABLE 4

EFFECT OF I-CELL REMOVAL ON CENTER FORMATION

	I-CELLS REMOVED		I-CELLS NOT REMOVED	
EXP.	Drops with Centers	Drops Without	Drops with Centers	Drops Without
A	0	4	3	1
B	1	5	3	3
C	0	8	4	2
Total	1	17	10	6

use. Replicate drops containing 500 myxamoebae were dispensed by loop on washed agar as described in section D. Within 2 minutes after deposition, each drop was examined. Where present, I-cells were immediately removed. As seen in Table 4, of a total of 18 drops so treated, only one produced an aggregate, whereas, of 16 control drops, 10 aggregated, yielding a normal incidence of 63 per cent. The probability that this difference could be equaled or exceeded by chance is less than 0.001 by chi-square test, 0.023 by Student *t*-test.

It is interesting that in similar experiments, in which the I-cells were washed only three times, were not kept in an ice bath prior to use, and in which the cells were removed somewhat less rapidly, the incidence of centers in the experimental drops was 26 per cent or slightly less than half of that in the controls.

F. *The Ability of I-Cells To Induce Aggregation.*—This capacity was examined by adding I-cells to test populations of 200–250 myxamoebae in order to determine whether the addition would increase the incidence of aggregative centers. The addition of randomly selected cells at the same state of development served as a control.

Washed myxamoebae were dispensed on 6× washed agar at a density of about 150 cells/mm^2. After 20 minutes, enough of the excess fluid had been absorbed by the agar to permit accurate microscopic examination. I-cells were identified and were moved to the test areas, using a glass loop mounted on a deFonbrunne micromanipulator. The test areas had been prepared as follows. Washed myxamoebae were dispensed on 9× washed agar at a density of 250 cells/mm^2. After 1 hour, at

which time all the excess fluid had been absorbed, 1-mm² areas were delineated by scoring the agar surface at the center of each drop with parallel razor-blade fragments mounted 1 mm. apart. The cells outside each square were brushed away, thus leaving populations of 250 cells at a density slightly above optimal. On 9× washed agar, the myxamoebae do not spread appreciably beyond the confines of their initial positions but can aggregate normally.[1]

As mentioned, the ratio of centers formed to cells present at optimal density has been found to be 1:2,200 for *D. discoideum* wild type. Thus one would expect that replicate populations of 250 cells would yield a Poisson distribution of centers with a frequency of about 11 per cent. The frequency actually observed was 12.1 per cent (last column of Table 5, experiments 1–7). The first two columns of Table 5 show to what extent the addition of I-cells and randomly selected cells affected this frequency. The data were obtained from 10 experiments performed after the test system and micromanipulative techniques had been perfected.

TABLE 5

INDUCTION OF AGGREGATES BY I-CELLS*

	I-CELLS			RANDOM CELLS			BACKGROUND		
EXP.	Squares	Centers	Per Cent	Squares	Centers	Per Cent	Squares	Centers	Per Cent
1	10	6	60				124	14	11
2	7	5	62				53	6	11
3	15	8	53				32	2	6
4	11	7	64				18	4	22
Total	43	26	60.5				227	26	11.4
5				12	2	17	31	4	13
6				18	1	6	24	3	13
7				23	4	17	24	4	17
				53	7	13.2	79	11	13.9
8	6	2	33	13	0	0	30	1	3.5
9	10	3	30	16	1	7	64	1	2.6
10	7	3	43	10	0	0	65	1	1.5
Total	23	8	34.7	39	1	2.5	159	3	2.0

* See text for details.

Experiments 1–7 indicate that the addition of randomly selected cells did not affect the incidence of aggregative centers but that the addition of I-cells raised the frequency to 60.5 per cent over-all, 72 per cent being the highest value achieved in a single experiment. This is particularly impressive when one considers that the act of transfer undoubtedly did considerable damage. About 10 per cent of the I-cells (but none of the randoms) lysed in transit as a result of surface-tension effects in the loop or mechanical damage during deposition. The remainder rounded up after transfer and did not assume the normal I-cell shape until about 2 hours had passed. Moreover, preliminary attempts to grow clones from I-cells after manipulation to fresh agar have yielded only a 75 per cent viability of deposited cells against a random cell viability of precisely 100 per cent in 20 trials of each type. Consequently, the value of 60 per cent is undoubtedly a low estimate of the initiative capacity of I-cells.

The difference between the I-cells and randomly selected individuals was dramatically revealed in three other experiments (8–10) in which, because the population size and density of the test areas were too low (about 150 cells/mm²), a submaximal number of centers was formed. In these experiments the background fre-

quency was 2 per cent; the random cell frequency, 2.5 per cent; the I-cell frequency, 34.7 per cent, seventeen-fold greater than background.

Withal, it can be said that a clear phenotypic difference exists between the I-cells and the remainder of the population with respect to initiative capacity. This, of course, does not preclude the possibility that another cell type, unrecognized by us and present in small proportion, might not also possess a high degree of initiative capacity. Moreover, the background frequency against which we have had to work permits us to say only that the incidence of cells that can initiate centers in the randomly selected population must be considerably less than 1 out of 10, while that in I-cells must be at least 6 out of 10 and is very probably higher.

DISCUSSION AND SUMMARY

The data presented here demonstrate that the I-cell does possess the capacity to initiate center formation and suggest compellingly that it is, in fact, *the* initiator cell for slime mold aggregation. Thus the I-cells could induce aggregates among test populations to which they had been added. The proportion of I-cells was about 20 per cent in excess of the number of centers that could be formed at optimal density. A high correlation was encountered between the positions of I-cells and of centers subsequently established. In small-population samples displaying a Poisson distribution of aggregates, there was an almost perfect correlation between the presence or absence of I-cells and of aggregative centers. Finally, removal of I-cells at a very early stage wiped out the capacity of populations to form aggregates. It seems fair to us on these bases to conclude that, if any alternative mechanism of center formation does exist, it plays an insignificant role under the conditions employed.

It is interesting that two other workers[8, 9] have observed the presence of abnormally large cells in fixed, stained smears of aggregating myxamoebae. Because the cells were dead, it is impossible to determine whether these correspond with the I-cells in properties other than size. However, the fact that the mean sizes of I-cells are so different and show much more limited intragroup fluctuation prompts us to believe that the large cells of Wilson and Skupienskii and the I-cells are the same. Wilson has suggested that the large cells are zygotes, that all myxamoebae engage in syngamy before entering the aggregate, and that the first-formed zygotes become the centers of aggregations. Fatal arguments have been raised against this interpretation as a whole,[1, 10] and Wilson has in fact modified[11] his original statements about the mandatory accompaniment of syngamy to the fruiting process while still maintaining that the large cells are zygotes. It should now be possible to submit this point to crucial examination. In this connection, preliminary data reveal that the I-cells are already present during the late lag and early lag phases and do not arise *de novo* during aggregation.

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† N.I.H. Post-Doctoral Fellow 1957–58.

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THE SONIC FRAGMENTATION OF COLLAGEN MACROMOLECULES

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Introduction. It has been shown that collagen macromolecules obtained by extracting collagenous tissues from diverse sources with citrate buffer are essentially monodisperse proteins having in common a high molecular asymmetry.[1–4] For example, soluble calf-skin collagen, termed "procollagen" by Orekhovich, has been shown to be 3100 A in length, 13.5 A in diameter, and to have a molecular weight of 360,000.[4] These macromolecules in solution consist of three polypeptide chains apparently held together with hydrogen bonds in the configuration of Rich and Crick (Collagen I). This highly organized structure disappears in a phaselike transition upon raising the temperature; in the case of soluble calf-skin collagen this thermal denaturation temperature is about 36° C.[4] Various kinds of precipitating conditions cause the collagen molecules to form long fibrils with periodic band patterns.[5] One of these is the one found in native collagen, having a periodicity of about 700 A. The other two have a periodicity approaching the length of the macromolecule, that is, nearly 3000 A.

Having found that deoxyribosenucleic acid, which consists of two molecular chains hydrogen-bonded together, could be broken by sonic irradiation into a series of lower-molecular-weight samples without damaging the native, highly organized structure, we were tempted to see whether collagen molecules could be similarly fragmented.[6] A successful outcome was not clearly predictable because the thermal denaturation temperature is relatively low and local heating in the solution may be sufficient to cause denaturation. The detection of denaturation is easy because soluble calf-skin collagen is characterized by a specific rotation of −415°, whereas the denatured collagen (parent gelatin) has a specific rotation of −135°.[4]

Preliminary experiments showed that exposure to 9-kilocycle sonic waves caused a continuous fall in intrinsic viscosity accompanied by only a very small change in optical rotation. This clearly indicated that the molecular weight was being decreased by fracturing of the collagen molecules but that their secondary structure

was largely preserved. It therefore seemed of interest to investigate such sonic fragments over a substantial molecular-weight range in order to see whether the rodlike character was strictly preserved and whether fracture might occur at selected points such as that corresponding to the 700 A periodicity. Further stimulus was provided by Hodge and Schmitt's finding that these sonic fragments formed new types of banded structures that reveal new aspects of the molecular morphology of collagen.[7]

Experimental Details.—Soluble calf-skin collagen was prepared by extraction of finely divided calf skin in citrate buffer at pH 3.7, as previously described.[4] The physical properties of the preparation were similar to those previously reported: intrinsic viscosity 12.5 in units of 100 cc/gm and specific rotation, $[\alpha]_D = -415°$.

Eight 30-cc. aliquots of a stock solution of this preparation (0.26 gm/100 cc in 0.15 *M* citrate buffer, pH 3.7) were exposed to 9-kilocycle sonic waves in a 50-watt Raytheon magnetostriction generator for varying lengths of time. Since the extent of degradation depends on the volume and temperature as well as on the time of exposure, the volumes were kept constant and the temperature was maintained at 8°–11° C. by means of circulating cooling water.

The samples produced in this way, termed "sonicates," are listed in Table 1, together with various measurements to be discussed. The eight sonicates prepared

TABLE 1

MOLECULAR CONSTANTS OF COLLAGEN SONICATES

Time of Irradiation (Min.)	$[\alpha]_D$	$[\eta]$	Axial Ratio, a/b	$\beta \times 10^{-6}$	$s^0_{20,w}$	Molecular Weight	K_s*	$K_s/[\eta]$*
0	415	12.50	181	3.44	3.02	374,000	2.65	0.212
10	410	10.75	166	3.38	2.95	336,000	2.50	.232
40	420	8.65	147	3.37	2.87	297,000	2.27	.262
80	...	6.25	123	3.30	2.79	250,000	2.02	.324
120	407	4.95	108	3.25	2.68	217,000	1.82	.368
180	392	4.00	96	3.21	2.64	192,000	1.66	.415
240	398	3.25	85	3.16	2.59	170,000	1.54	.478
360	390	2.45	73	3.11	2.54	149,000	1.42	0.580
440	...	2.20	68	3.08	..	137,000†	...	...

* K_s expresses the concentration dependence of sedimentation constants according to the relation $s^0/s = 1 + K_s c$. The ratio $K_s/[\eta]$ generally has a value of about 1.6 for highly flexible, randomly coiled polymers and about 0.6 for the more extended polymers, such as cellulose derivatives. These results show that it has a dependence on axial ratio for rodlike macromolecules.

† This value was determined from the intrinsic viscosity with the use of Fig. 1.

in this fashion involved irradiation up to 440 minutes. The specific rotation of these sonicates fell gradually with time of exposure but did not exceed 10 per cent of the interval between native collagen and parent gelatin.

Results.—The intrinsic viscosity and the sedimentation constant at infinite dilution were determined for each sonicate by making the relevant measurements as a function of concentration in the same citrate buffer solvent. The plots of the reduced specific viscosity and the reciprocal of the sedimentation constant, $s_{20,w}$, against concentration are shown in Figures 1 and 2. The desired quantities, corresponding to the intercepts of these graphs, are listed in Table 1.

It is seen that the intrinsic viscosity, measured at 24.8° C., falls from 12.5 to 2.20, and the sedimentation constant from 3.02 to 2.54 in the range covered by these nine samples. By examining the sedimentation diagrams, it was evident that frag-

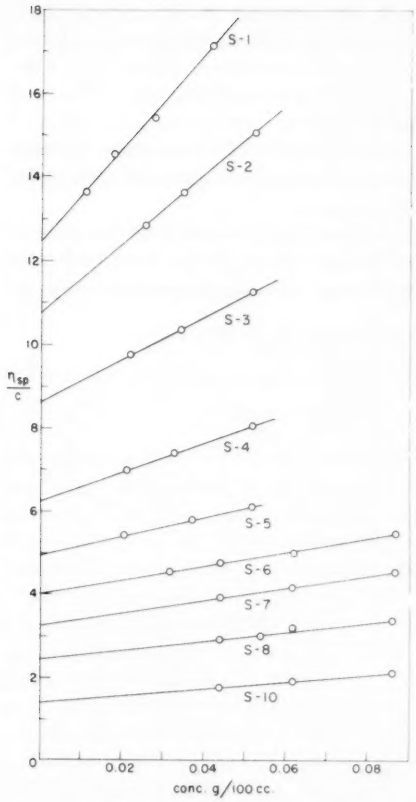

FIG. 1.—The reduced specific viscosity as a function of concentration for the collagen sonicates.

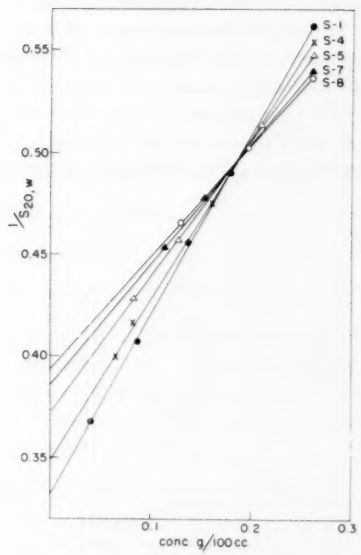

FIG. 2.—The reciprocal sedimentation constants as a function of concentration for the collagen sonicates.

mentation eventually affected all the molecules in the sample. For example, when the sedimentation diagram at 160 minutes (59,780 rpm) of sonicate 7, 8, or 9 was superimposed upon that for the original collagen under identical conditions, there was no overlap, despite the relatively small difference in sedimentation constants.

Molecular Weight and Hydrodynamic Properties of the Collagen Sonicates.—In view of the negligible amount of denaturation produced in the sonicates, it appears very likely that the sonicates are homologous pieces of the native collagen molecules that retain the rigid, rodlike structure. If this is true, the molecules will behave hydrodynamically as ellipsoids of revolution, and the molecular weight can be computed from the viscosity and sedimentation values, using the Scheraga-Mandelkern equation;[8]

$$M = \frac{s^0 n^{1/3}}{[\beta \quad (1 - \bar{v}\rho)]^{2/3}} \qquad (1)$$

In this equation η_0 (= 1.095 centipoise) is the viscosity of the solvent; N is Avogadro's number; $\bar{v}$ is the partial specific volume of collagen, which we have found to be 0.73; ρ (1.015) is the solvent density; and β is a parameter whose value is determined by the axial ratio of the hydrodynamically equivalent ellipsoid. This axial ratio is in turn determined by the intrinsic viscosity.

In Table 1 the axial ratio, a/b, is listed with each value of the intrinsic viscosity and next to this the value of β.[8] Substitution into the above equation then yields the molecular weight; this is listed in the next column.

The dependence of intrinsic viscosity and sedimentation constant on the molecular weight can now be examined. The results are shown in the form of double logarithmic plots in Figures 3 and 4. In the case of intrinsic viscosity (Fig. 3)

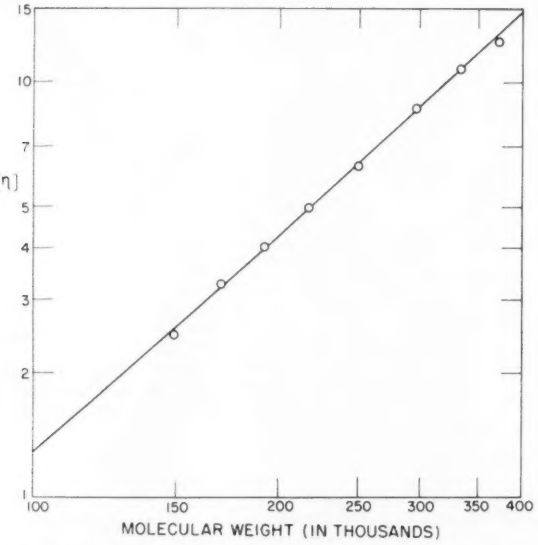

FIG. 3.—A double logarithmic plot of intrinsic viscosity against molecular weight. The line is a plot of Simha's equation with $2b$ = 16.7A and v = 0.73.

the dependence is very high. It can be represented empirically by the equation

$$[\eta] = 1.23 \times 10^{-9} M^{1.80} \quad (2)$$

This value of the exponent is that of the upper limit for ellipsoids having a constant minor axis or for cylinders of constant diameter, according to Simha's equation. The fit is excellent and confirms our assumption concerning the homologous, rod-like nature of the collagen sonicates.[10] The value used for the minor axis is quite acceptable. To convert it to the diameter of a cylinder of equivalent length and density, it must be multiplied by $(2/3)^{1/2}$: this yields 13.6 A. This value is in exact agreement with that previously found for collagen: it is slightly greater than the 12.0 A value derived from equatorial reflections in X-ray diffraction, as would be expected for a solvated molecule.

The dependence of sedimentation constant on molecular weight is small. Empirically it can be represented by (dashed line in Fig. 4)

$$s^{\circ}_{20,w} = 0.232\ M^{0.20}. \tag{3}$$

This likewise approximates the lower limiting value of the exponent for ellipsoids of constant minor axis. Perrin's equation[11] expresses the relation between s^0 and the axial ratio; for axial ratios greater than 10 it takes the following form:

$$s^0 = \frac{(1 - \bar{v}\rho)}{\eta_0\, \bar{v}} \frac{2}{9} b^2 \ln 2 \frac{a}{b}. \tag{4}$$

The axial ratio is related to the molecular weight of the collagen sonicates by

$$M = (4\ \pi/3)(a/b)b^3(N/v) = 2020\ (a/b). \tag{5}$$

Substitution for the constants in Perrin's equation leads to

$$s^0 = 1.16 \log 2\ (a/b) = 1.16 \log\ (M/1010). \tag{6}$$

The solid line drawn in Figure 4 results from equation (6). The slope of 1.22 in the empirical line is a little larger than 1.16 in the theoretical one. This discrepancy, however, is within experimental error. Thus it is seen that the sedimentation constants, as well as the intrinsic viscosities, vary with molecular weight in the manner expected of homologous, rodlike molecules with variable length.[12]

The Molecular-Weight Limit in Sonic Fragmentation.—If the rate of molecular scission, that is, sonic rupture of the collagen molecules, were directly dependent on the time of irradiation, the molecular weight would fall with time in such a manner that the reciprocal of the molecular weight would be linear with time. When the data in Table 1 are plotted in this manner, one observes such a linear relation for the first 120 minutes, but thereafter the rate of increase of the reciprocal molecular weight diminishes. This behavior is widely observed in the sonic degradation of polymers. Several analyses of this behavior have been based on the assumption that there is a lower limit of molecular weight below which sonic irradiation will not produce scission. Schmid's[13] formulation of the problem is convenient for our purposes. He assumes that the rate of scission is proportional to the difference between the molecular length at a given time and the limiting molecular length beyond which

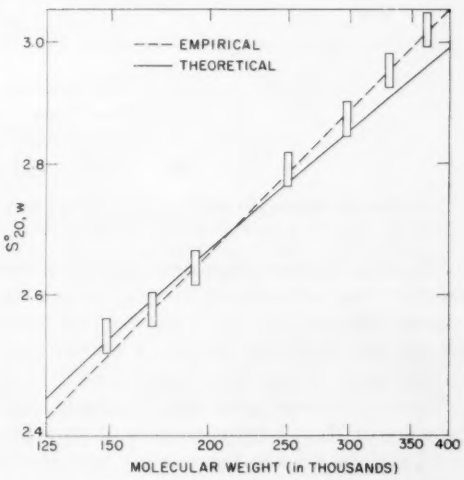

FIG. 4.—A double logarithmic plot of sedimentation constants at infinite dilution against molecular weight. The solid line is a plot of Perrin's equation; the dashed line is a plot of empirical values.

scission will not occur. Since length is proportional to molecular weight, we may denote M_0 as the initial molecular weight, M_t as the molecular weight at time t, and M_f as the final or limiting molecular weight. His analysis then takes the form

$$\frac{M_f}{M_t} + \ln\left(1 - \frac{M_f}{M_t}\right) = -\frac{KM_f^2}{cm^2}\,t + \frac{M_f}{M_0} + \ln\left(1 - \frac{M_f}{M_0}\right), \qquad (7)$$

where c is the initial concentration of the polymer in moles per liter and m is the molecular weight of a monomeric unit.

To explore the feasibility of this explanation of our data, we have plotted the left side of equation (7) against time for various values of M_0 relative to M_f. When M_f is taken as $^1/_4\ M_0$, a linear plot, shown in Figure 5, is obtained. Choices of

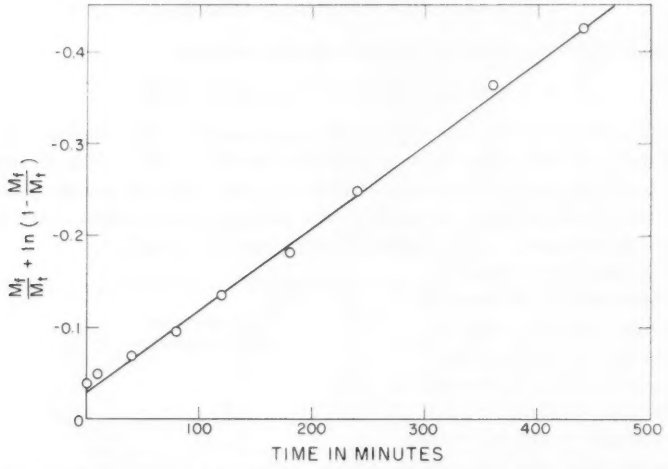

FIG. 5.—A plot of time-dependence of the molecular weight upon exposure to sonic waves in terms of the Schmid equation.

$^1/_2\ M_0$ or $^1/_8\ M_0$ show clear deviations from linearity in opposite directions. This indicates that our data are at least compatible with the idea that one-fourth the original collagen molecule is the limiting molecular weight produced by scission. To test this possibility further, a sample was prepared by very long sonication, 1,140 minutes. From its intrinsic viscosity (1.405) and the plot of Simha's equation in Figure 3, its molecular weight was estimated to be 105,000. On the other hand, the value of M_t at 1,140 minutes' sonication predicted by equation (7) with $M_f/M_0 = {}^1/_4$ and the slope in Figure 5 ($= -(KM_f^2/\text{cm}^2) = 0.912 \times 10^{-3}$/minute) was 108,000.

Thus it appears that the time-dependence of the molecular weight of collagen exposed to sonic irradiation can be explained in terms of scission being most probable at three evenly spaced regions of the molecule. Since this would correspond to the 700 A periodicity observed in native collagen fibrils, it is tempting to suggest that the molecular regions giving rise to the 700 A spacing are the points where the collagen molecule is most easily broken. However, at this stage this must be considered only as a plausible suggestion. Electron-microscopy and flow-birefringence

studies will provide a critical test. Meanwhile, it is relevant to point out that the molecular lengths of 45 and 55 per cent of the over-all length of the original collagen molecule, found by Hodge and Schmitt[7] in their electron-microscopic study of collagen sonicates is consistent with this suggestion that there is a preferential point of scission located at (approximately) the mid-point as well as at one-quarter and three-quarters of the distance along the original collagen macromolecule.

Summary.—The exposure of soluble calf-skin collagen to 9-kilocycle sonic irradiation at low temperature causes fragmentation of the long, rodlike collagen macromolecules into shorter pieces that retain the three-stranded, helical structure. A series of samples produced by varying the time of exposure from 10 to 440 minutes were found to have molecular weights in the range of 335,000–140,000. The intrinsic viscosity of these samples depended on the 1.80 power of the molecular weight, and the sedimentation constant on the 0.20 power. This dependence was compatible with the behavior expected of homologous, rodlike molecules of 13.6 A diameter (solvated). The time-dependence of the molecular-weight change is compatible with preferential fracture of the collagen macromolecule into halves and quarters.

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*INTERACTION PROPERTIES OF SONICALLY FRAGMENTED COLLAGEN MACROMOLECULES**

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Nishihara and Doty[1] have shown by physical-chemical measurements that sonic irradiation of soluble collagen (tropocollagen,[2] abbreviated "TC") results in fragmentation of the long rodlike macromolecules into shorter pieces which retain the three-chain helical structure characteristic of the native macromolecules.[3] Thus, while sonic irradiation reduces the viscosity of such solutions very considerably, their optical rotation is affected only slightly. Nishihara and Doty concluded that the time-dependence of the molecular-weight change is compatible with a preferential fragmentation of the collagen macromolecules into halves and quarters. It will be shown here that electron-microscopic examination of ordered aggregates produced from irradiated collagen solutions confirms, in general, the above conclusions.

One of the most striking properties of native collagen macromolecules is their ability to aggregate in highly characteristic patterns, depending on the chemical environment to which they are exposed. These aggregation states are characterized by highly specific band patterns, as seen in the electron microscope. The band structure is best seen when the specimens are treated with an "electron stain," such as phosphotungstic acid (PTA). The capacity to form such ordered structures is lost when the TC is denatured. It seemed, therefore, that application of this electron-microscope technique could test the correctness of Nishihara and Doty's conclusions and might also yield information concerning the specific points along the TC macromolecule at which scission occurs. This has proved to be the case, and, in addition, the experiments have yielded information concerning the properties of the end-regions of the TC macromolecules in relation to fibrogenesis and to the formation of ordered aggregates in general.

The results clearly demonstrate that sonic fragmentation of TC is not a random process but rather involves scission at well-defined loci, identifiable by analysis of the band patterns of segment long-spacing (SLS.) type aggregates.[2] In this type of ordered structure, produced by addition of adenosine triphosphate (ATP), the structurally asymmetric macromolecules are in parallel array, and the band pattern is, in effect, a kind of molecular "fingerprint." The results also show that sonic irradiation produces profound effects other than scission of the macromolecules. Of these, the most important is a rapid alteration of "end-regions" without appreciable change in length of the macromolecules. This effect is strikingly manifested in an alteration of the end-to-end aggregation properties of the TC. Thus sonic irradiation rapidly gives rise to a heterogeneous population of macromolecules, whose diversity resides not only in particle length but also in end-to-end polymerization properties. Such changes in macromolecular "end-regions" are probably not detectable by physical-chemical means. However, it should be borne in mind that the electron-microscopic techniques reveal information only about those TC macromole-

PLATE I

(The black bars represent 1 μ except where otherwise indicated)

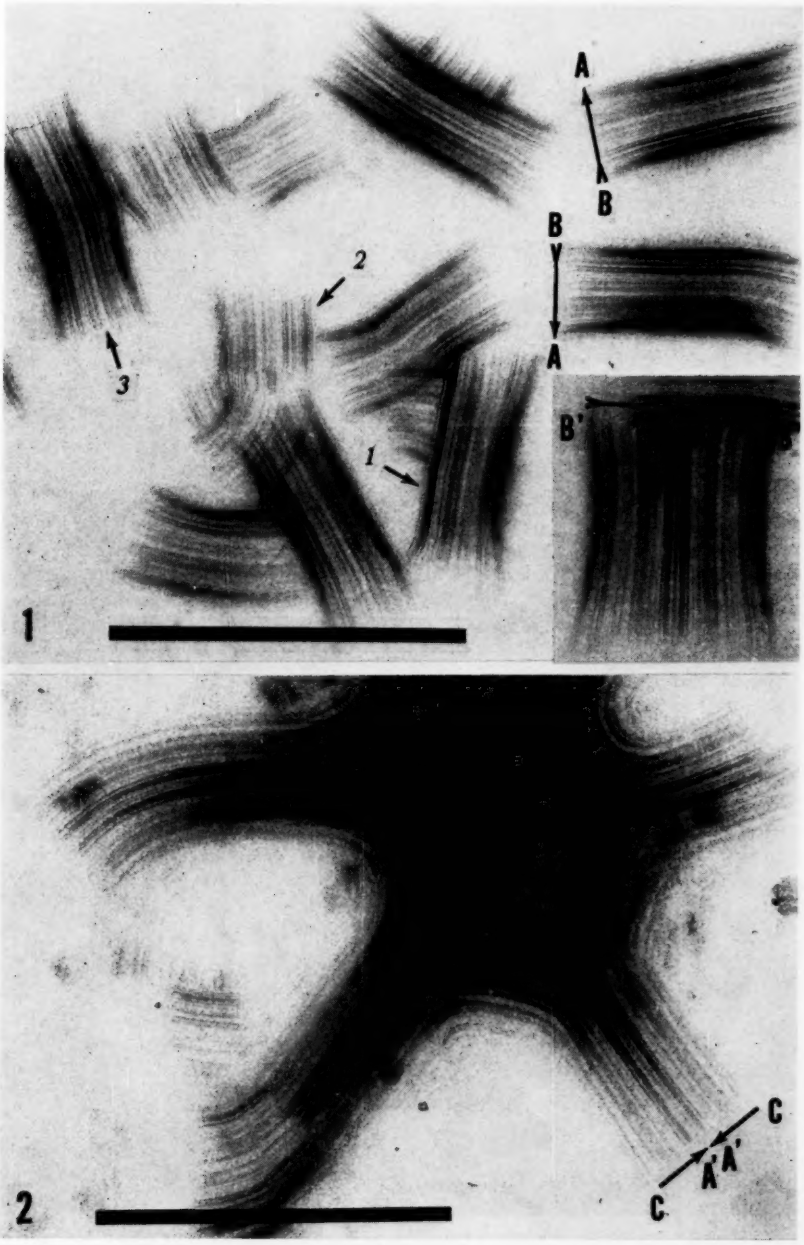


FIG. 1.—Whole monomeric SLS-type aggregates from a solution irradiated for 20 minutes, showing a relatively high degree of lateral aggregation. Arrows labeled *A*, *B* indicate the macromolecular polarization. Inset shows a dimeric form of the type *B'A'–A'B'*. See text for explanation of arrows *1*, *2*, and *3*. ×55,000.

FIG. 2.—SLS aggregates of the fragment dimeric type *CA'–A'C* from a solution irradiated for 240 minutes, showing very extensive lateral aggregation in the form of "ribbons." ×55,000.

PLATE II

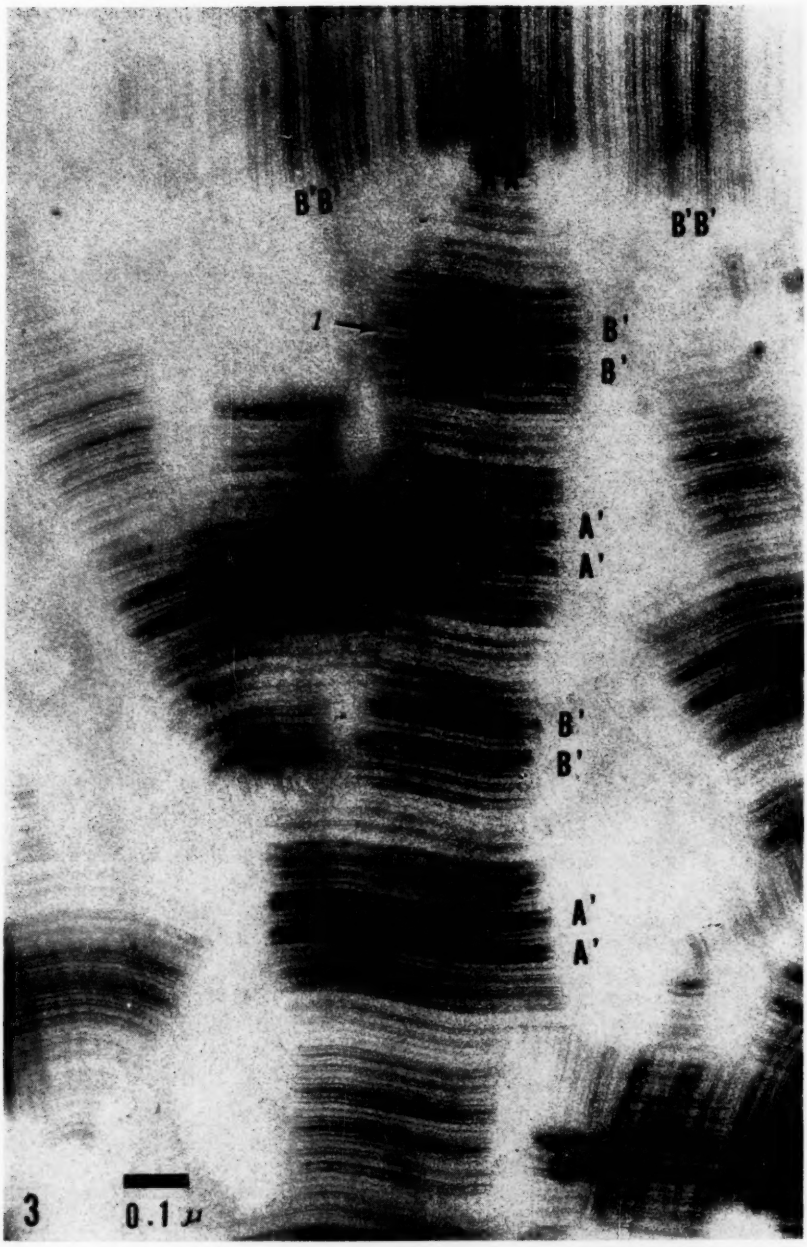


FIG. 3.—SLS aggregates of whole polymeric type from a solution irradiated for 240 minutes, with some isomorphous growth of fragments. Note the detailed band structure, particularly in the A'–A' and B'–B' junctions. ×100,000.

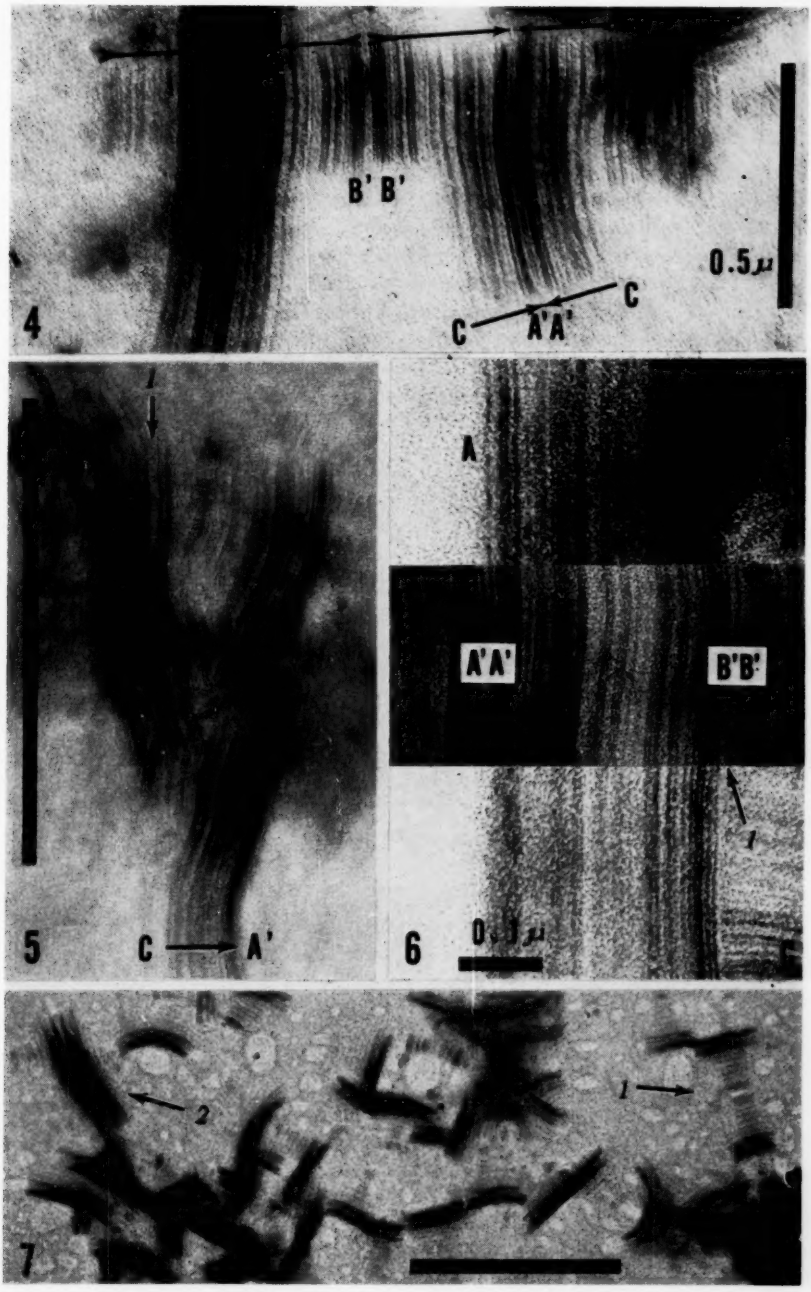


FIG. 4.—SLS aggregate of whole polymeric type, showing extensive isomorphous growth of the fragment dimeric form CA'–$A'C$, 240 minutes' irradiation. ×75,000.

FIG. 5.—SLS aggregates from 240-minute solution, showing formation of ribbon-like fragment monomeric forms of the type $A'C$. Arrow *1* indicates the prominent F–G interband (compare with Fig. 1, arrow *3*). ×71,000.

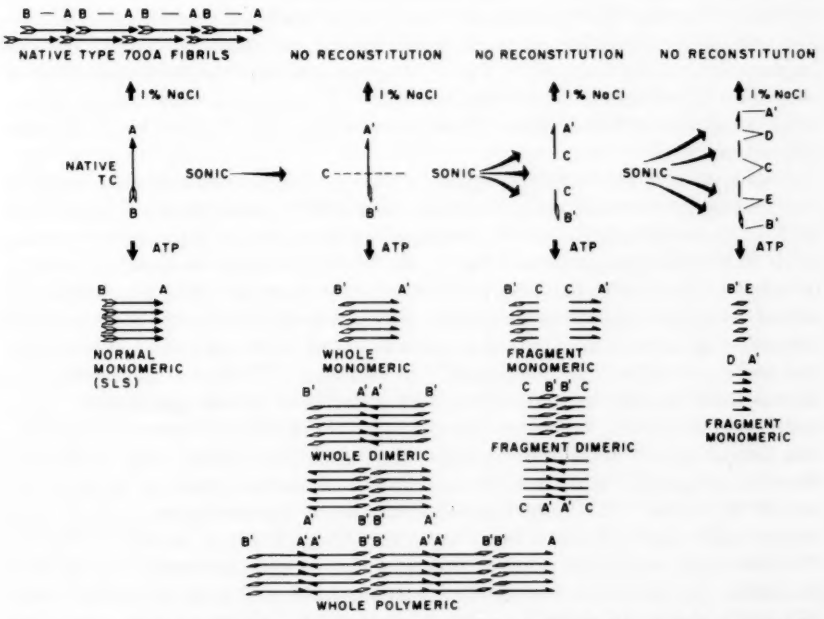
FIG. 6.—Composite micrograph for comparison of band structures in whole monomeric SLS forms (*a* and *c*) with the pattern in the whole polymeric form (*b*). Arrow *1* indicates the new band arising from polymerization in the B'–B' junction. ×130,000.

FIG. 7.—SLS-type aggregates formed from a TC solution irradiated for 335 minutes. Most of the structures are fragment monomeric forms with widths corresponding to particle lengths of about one-fourth that of the intact TC macromolecule. ×33,000.

cules or fragments capable of forming ordered aggregates under a particular set of conditions. The fraction of the molecular population capable of such precipitation decreases with time of irradiation.

Samples of soluble collagen in dilute acetic acid, irradiated for times ranging from zero to 335 minutes,[5] were tested for their ability to form native-type (700 A repeat) fibrils by dialysis against 1 per cent NaCl solutions and SLS-type aggregates by addition of ATP (final concentration 0.2 per cent). The resulting precipitates were mounted on carbon films, stained with 0.2 per cent PTA buffered to pH 4.2, and examined in a Siemens Elmiskop Ia electron microscope. The amounts of precipitable protein in each sample were determined by the Lowry method,[6] and results were checked by estimation of the protein concentration of the supernates.

Results.– The results are shown schematically in test Figure 1. The native tropo-



TEXT FIG. 1.—Diagram to illustrate the main effects of sonic irradiation on the tropocollagen macromolecule

collagen macromolecule, having dimensions[7] of about 14 × 3000 A, is represented by an arrow with *A* and *B* ends, symbolic of the intrinsically asymmetric distribution of amino acid residues along its length. This asymmetry is best revealed in the polarized band pattern of SLS aggregates, in which the TC macromolecules are considered to be in register, with like ends "pointing" in the same direction, i.e., in parallel array.

The effects of sonic irradiation on soluble collagen solutions are best considered in terms of the occurrence of several different processes proceeding concurrently but at different rates. The most rapid of these processes is an impairment of the capac-

ity of the irradiated solutions to form fibrils of native type (700 A repeat). As indicated diagrammatically in text Figure 1, formation of native-type fibrils most likely involves packing of protofibrils (each of which may be regarded as a linear polymer of TC arising from end-to-end interaction of the *A–B* type) in a way such that adjacent protofibrils are displaced longitudinally with respect to one another by one-quarter of the macromolecular length. The native type of aggregation is the first to be affected by sonic irradiation. Thus for a control value of 100 per cent (corresponding to *ca.* 86 per cent protein precipitation on dialysis against 1 per cent NaCl), 80 minutes of irradiation reduced the amount precipitated to 33 per cent. After 240 minutes, the amount precipitated was essentially zero. In contrast to these results, the amounts precipitated with ATP were 78 and 28 per cent of the control, respectively, for the 80- and 240-minute samples, the control value corresponding to about 93 per cent protein precipitation. Since electron-microscopic examination of the ATP precipitates from briefly irradiated solutions (20–80 minutes) revealed only whole monomeric, whole dimeric, and whole polymeric SLS-type aggregates (text Fig. 1 and Pl. I, Fig. 1), it seems clear that the most rapid effect of sonication is exerted on the end-regions of the TC macromolecules without appreciable alteration of their lengths. This is indicated in text Figure 1 by designating the ends *A′* and *B′*.

Examination of the SLS-type aggregates has yielded information very useful in interpreting the effects of sonic irradiation on the TC macromolecule. Addition of ATP to a control solution of TC results in the formation of typical whole monomeric SLS-type aggregates (text Fig. 1), the widths of which are usually less than, or equal to, the molecular length. Dimeric or polymeric forms are seldom observed. However, following irradiation sufficient to inhibit for the most part the formation of native-type fibrils (i.e., destruction of *A–B* end-to-end interaction), two pronounced effects are noted in ATP precipitates. The first of these reflects an increased side-to-side interaction (i.e., higher degree of lateral aggregation) and is manifested in a greatly increased segment width (Pl. I, Fig. 1). Second, as irradiation progresses and in solutions in which the degree of fragmentation is still small, there is a progressive increase in the amount of end-to-end interaction of the A′–A′ and B′–B′ types. This results in the formation of increasing amounts of whole dimeric and whole polymeric forms (text Fig. 1; Pl. I, Fig. 1, and Pl. II, Fig. 3). The effects of sonication prior to fragmentation of the macromolecules are thus threefold: (1) inhibition of end-to-end interactions of *A–B* type, (2) enhancement of homologous end-to-end interactions of A′–A′ and B′–B′ type, and (3) an increase in the degree of lateral aggregation.

With longer irradiation times, scission of the TC macromolecules becomes obvious in the reconstitution forms (Pl. I, Fig. 2, Pl. II, Fig. 3, Pl. III, Figs. 4, 5). The fragmentation is not random but occurs initially in a well-defined region near the point previously designated as the "*G*" band[4] (readily visualized because of the easily recognizable *F–G* interband) (see arrow *3*, Pl. I, Fig. 1, and arrow *1*, Pl. III, Fig. 5). The initial scission products (*A′C* and *B′C*) are thus about 55 and 45 per cent, respectively, of the original TC length, a result in rough agreement with the estimates of Nishihara and Doty[1] from physical-chemical measurements. Such fragments are capable of forming monomeric (Pl. III, Fig. 5), and dimeric (Pl. I, Fig. 2) SLS aggregates. In addition, the fragments are capable of isomorphous

growth on whole polymeric SLS aggregates (Pl. II, Fig. 3, and Pl. III, Fig. 4), with the band structures in exact register.

A point of considerable importance, illustrated in text Figure 1, is that end-to-end polymerization of scission products apparently never involves ends produced by the fragmentation but only the original ends of the macromolecules. Thus, when scission occurs at point *C* (text Fig. 1), dimeric or polymeric forms, involving end-to-end linkage of the *C*–*C* type, are never observed. In contrast to this is the high frequency of occurrence of *A'*–*A'* and *B'*–*B'* types; occasional aggregates of *A*–*B* type are also seen. It seems, therefore, that the original ends of the macromolecules possess properties which cannot be duplicated in new ends produced by scission (presumably involving simultaneous breakage across the entire triple helix structure).

Fairly extensive irradiation (order of 5 hours or more) produces smaller fragments of the type arising from scission at the points *D* and *E* (text Fig. 1). Of these scission products, only those including *A'* or *B'* ends are capable of forming SLS-type structures, seen as characteristically long "ribbons" (Pl. III, Fig. 7). Their dimensions correspond to particles with lengths about one-quarter that of the intact TC. Arrow *1* in Figure 7 indicates a whole dimeric form with isomorphous growth of *A'D* particles; arrow *2*, a fragment dimeric form of the type *CB'*–*B'C*. A notable feature is the absence of dimeric forms arising from particles of the type *A'D* or *B'E*. It seems necessary to conclude, therefore, that extensive irradiation results not only in the production of particles about one-quarter of the original molecular length but also in a further modification of the *A'* and *B'* ends.

Discussion.—The specific band patterns observed in ordered aggregates derived from sonically irradiated solutions of TC, described above, have been tentatively interpreted in the following manner. It is assumed that the band patterns characteristic of the various TC aggregation states are e.g., native type with 700 A repeat, fibrous long-spacing (FLS), and SLS—arise from interaction between adjacent macromolecules through certain types of amino acid side chains situated at specific locations along each of the three polypeptide chains which coil about each other in the triple helix configuration characteristic of native TC. The particular band pattern observed depends on the packing of the TC in one of a number of different ways, viz., parallel (SLS), antiparallel (FLS), and the staggered array (native type). Bands are considered to be formed only by interaction of regions of the macromolecules which manifest the native helical coiling. Denatured collagen, in which the chains have become uncoiled (as indicated by reduced optical rotation), does not form banded aggregates.

The formation of SLS aggregates by ATP has been ascribed to modification of the interaction properties of TC resulting from a combination of ATP, probably for the most part with the side chains of the lysine residues.[2] It is of interest to consider some of the present results in the light of this concept. As described above, only those TC fragments which include an original TC end-region appear to be capable of lateral aggregation to form SLS-type structures. It is reasonable to conclude, therefore, that the majority of the ATP binding sites are concentrated in end-zones of the TC macromolecules. Similarly, it is clear from the high density of band structure in the end-regions of whole monomeric SLS forms (Pl. I, Fig. 1) that the sites which preferentially bind phosphotungstic acid are similarly concentrated

near the ends of the TC macromolecules. The comparison is interesting, for the bands observed in PTA-stained specimens have been ascribed to the presence of bulky side groups which allow accumulation of PTA in interstitial spaces[8, 9] or to a relatively specific combination with the guanidino groups of arginine.[10] In any event, it is clear that the basic amino acids are involved importantly in both ATP and PTA interaction and are concentrated to a considerable extent in the end-regions of the TC macromolecule. It is of importance, therefore, to gain information concerning the terminal architecture of the TC macromolecule.

Since the discovery of the SLS type of aggregation pattern,[2] it has been noted that the ends of the segments are relatively dense and the bands poorly resolved in these regions. However, in a few instances and particularly in lightly irradiated samples, we have been fortunate enough to obtain micrographs showing the detailed band structure in the end regions (e.g., Pl. I, Fig. 1, arrow *1* shows the structure in the *A* end; arrow *2*, that in the *B* end). In Plate III, Figure 6, the detailed band patterns in the end-regions of the monomeric SLS form are compared directly with the pattern present in a polymeric form involving *A'*–*A'* and *B'*–*B'* linkages. At the *A* end (Pl. III, Fig. 6, *a* and *b*), the two bands in the polymeric structure corresponding to the terminal band of the *A* end in the monomeric form are separated by an interband and are about 100 A apart. In the case of the *B'*–*B'* junction, the two corresponding terminal bands are separated by about 180 A, and, in addition, a new band (arrow *1* in Pl. III, Fig. 6, *c*) not present in the monomeric form appears in the middle of the junction. On the assumption that the band pattern of monomeric SLS arises only from those regions of the TC macromolecules possessing the triple helix configuration, the above observations strongly suggest the presence of chain appendages at both ends of the TC macromolecule with maximum lengths of about 100 and 200 A for the *A* and *B* ends, respectively. This conclusion is strengthened by the observation that molecular ends produced by scission of TC are unable to form dimeric or polymeric forms involving end-to-end interaction of the type *C*–*C* (text Fig. 1). It is relevant to note that, following the demonstration of the triple helix structure of collagen, Boedtker and Doty[7] deduced, from physical-chemical measurements, the presence of terminal dangling chains at one or both ends of the TC macromolecule. The presence of such structures would also be consistent with the observation of Doty and Nishihara[11] that the three component chains of the TC macromolecule are approximately equal in length. Thus the lengths of the single- (or double-) chain appendages extending beyond the main triple helix part of the macromolecule would depend on the relative positioning of the three chains with respect to one another.

It is clear from the electron micrographs of polymeric SLS forms (Pl. II, Fig. 3, and Pl. III, Fig. 6, *b*) that the regions occupied by such terminal chains exhibit little affinity for PTA (with the exception of the new band formed in the center of the *B'*–*B'* junction and indicated by arrows labeled *1* in Figs. 3 and 6). It seems probable, therefore, that the amino acid composition of the terminal chains resembles that of interband regions of the body of the TC macromolecule in lacking the concentrations of the basic amino acids presumed to exist in the band regions. This possibility would be consistent with the amino acid composition observed in certain peptides derived from collagen.[12]

Continuing this analysis, it seems logical to postulate that end-to-end aggregation

or polymerization of TC macromolecular monomers may involve a specific type of coiling of the terminal dangling chains about each other to form a highly ordered structure. No X-ray diffraction evidence concerning the structure of these junctional regions is available, but the detailed band structure of the polymeric SLS forms, particularly the appearance of the new band in the center of the B'–B' junctions, is consistent with such an interpretation. This inference is of considerable interest, for Doty and Nishihara[11] have noted that, for three different types of collagen, the difference between the thermal shrinkage temperature and the denaturation temperature in solution is essentially constant (about 29 deg) and is independent of the hydroxyproline content. This temperature difference may in a sense be regarded as the "crystal energy" of the collagen fibrils. It seems reasonable to suppose that a part of this energy represents energy of interaction (or, more specifically, a "coiling energy") of the postulated terminal chains. The characteristic shrinkage and denaturation temperatures of various collagens reflect differences in amino acid composition, particularly the content of hydroxyproline,[13] within the main body of the TC macromolecule. The relatively constant difference between these characteristic temperatures in the three collagens examined by Doty and Nishihara may reflect a basic similarity of structure and possibly of amino acid array in the terminal chains of the TC. This possibility would be consistent with the similarity in aggregative behavior of TC obtained from various sources.

The nature of the interaction between the TC terminal chains is not certain, but, in view of the relative ease and the reversibility of TC aggregation phenomena, it seems probable that the bonds are relatively weak, probably chiefly hydrogen bonds. This would be consistent with the observations of Gross[14] that the thermal gelation of neutral salt solutions of TC is inhibited by low concentrations of urea and other substances capable of affecting hydrogen bonds. The insolubility of the macromolecules in collagen in certain types of connective tissue may be due in part to the formation of more stable bonds between the terminal chains of TC, as well as between side-chain groups along the body of the macromolecules, as has already been suggested.[15]

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*THE SOLUTION PROPERTIES AND CONFIGURATIONS OF A POLYAMPHOLYTIC POLYPEPTIDE: COPOLY-L-LYSINE-L-GLUTAMIC ACID**

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Evidence is accumulating to support the view that substantial and characteristic portions of many proteins exist in the α-helical configuration.[1-3] Indeed, optical rotatory and infrared spectral studies strongly suggest that this is the only widely occurring, periodic structure in proteins. This evidence has been obtained largely by using synthetic polypeptides, whose configuracions could be varied and accurately ascertained, to calibrate the physical methods which are configuration-sensitive.

The two water-soluble polypeptides that have been studied from the configurational point of view have been found to exist as complete α-helices when their charge has been sufficiently reduced. Thus poly-L-glutamic acid[4,5] becomes helical below pH 4.5 and poly-L-lysine above pH 10.5.[6] In the neutral range of pH, both these polypeptides exist as structureless, randomly coiled chains.

It is evident, therefore, that the next step in an effort to mimic protein structure, and perhaps function, in synthetic polypeptides requires the synthesis of polypeptides that will exhibit some helical configuration in the neutral pH range. For this reason, equimolar copolymers of L-lysine and L-glutamic acid have been made because it seemed likely that in this case the local charge density would be sufficiently reduced to permit the α-helix to exist in the pH range near neutrality. This is a preliminary report on the configurational properties of these copolymers from which it will be seen that this expectation has been borne out to some extent. Polypeptides similar to these have been investigated by Blout and Idelson with special reference to compositional effects and are being reported concurrently.[7] Previous to this, the only work on this kind of copolymer has been that of Shavit,[8] who made an electrophoretic study of copolymers of L-aspartic acid and L-lysine.

Synthesis.—This study is limited to equimolar copolymers of L-lysine and L-glutamic acid. These were prepared by first copolymerizing the N-carboxy-anhydrides of ϵ-carbobanzoxy-L-lysine and γ-benzyl-L-glutamate followed by removal of the protecting groups. Since the rates of polymerization of these two N-carboxy-anhydrides are different, it is to be expected that the incorporation of one residue will be greater than the other in the early part of the chain growth and the reverse will be the case in the later stages. The early results obtained in a study of the polymerization kinetics of this system by R. Salovey in this laboratory have

helped us minimize but not eliminate this effect. From his results we were able to choose conditions in which the anhydride consumption was linear throughout the observable region of the reaction. Fulfilling this condition insured that excessive clustering of each type of residue was avoided but the precise degree to which we approached randomness of sequence must be left until the completion of Salovey's work.

The procedure adopted consisted of polymerizing the N-carboxy-anhydrides of γ-benzyl-L-glutamate and ε-carbobenzoxy-L-lysine[10] at a total concentration of 4 g. per 100 cc. in dimethyl-formamide. Sodium methoxide, at a concentration of 0.5 mol per cent relative to the N-carboxy-anhydrides, was used to produce copolymers with molecular weights of about 100,000.

The product was precipitated with water, dissolved and lyophilized from dioxane, and converted to copoly-L-glutamic acid-L-lysine hydrobromide by stirring in glacial acetic acid saturated with dry hydrogen bromide and allowing to stand at 0° 0° C. for about eight hours.[11] This product was precipitated with ether, redissolved in methanol, precipitated with ether, dissolved in water and dialyzed against water. The resulting solution was shown to have no absorption in the 2580 A region showing that the protecting aromatic groups had been completely removed. Concentration was determined by nitrogen analysis, assuming 16.3 per cent N.

Characterization.—The polypeptide produced in this manner exhibited uniform solubility behavior only if they were first exposed to alkaline pH, e.g., pH 13, for several hours. When so treated they were completely soluble at all pH's provided the ionic strength was not too low (~0.1 M NaCl). In water or very low ionic strength some samples developed visible turbidity in the pH range 4.5 to 9.5. We adopted a standard procedure of alkaline treatment and used 0.15 M NaCl as a solvent.

When examined near pH 7 in 0.15 M NaCl, the samples were found to display a single peak in electrophoresis and sedimentation. In electrophoresis the reversal of the direction of migration occurred within 0.2 pH units and was located very close to 7.0. The amount of spreading during electrophoresis at the isoionic point was less than that for γ-globulin.

The intrinsic viscosities of the copolymers were about 0.30 when determined in 0.15 *M* NaCl near neutrality. For example, at this ionic strength and pH 6.2, one sample had an intrinsic viscosity of 0.25 and a sedimentation constant, $s^0_{20,w}$, of 1.93 *S*. Using a partial specific volume of 0.74 and a value of 2.6×10^6 for the constant in the Flory-Mandelkern equation, these data lead to a molecular weight of 36,000.

Equilibrium Behavior as a Function of pH.—If the configurational possibilities of the polypeptide chain are limited to the α-helix and the random coil, the relative proportions of these can be estimated from the specific rotation and more accurately assessed from the rotatory dispersion.[1-3] The specific rotation at 0.15 ionic strength as a function of pH is shown in Figure 1. For reference the corresponding behavior of poly-L-glutamic acid[5] and poly-L-lysine[6] are included. It is seen that the helical content of the copolymer is a maximum in acidic conditions, drops in the neutral range, and diminishes further in alkaline solution. Rotatory dispersion measurements place the helical content at 50 per cent in acidic solution ($b_0 = -310$, $a_0^H = 435$), 15 per cent at pH 7 ($b_0 = -100$, $a_0^H = 120$) and zero at pH 12.

With the nearly symmetrical behavior of poly-L-glutamic acid and poly-L-lysine with regard to helix formation as a function of pH, it is at first surprising to find a complete lack of symmetry about neutrality in the helical content of the copolymer. However, the explanation for this appears to reside in the observation that the poly-L-lysine helix is indeed weaker than the poly-L-glutamic acid helix and will not support as high a charge density.[6] It seems likely that hydrogen bonding between pairs of glutamic acid residues on adjacent turns of the helix may provide the additional increment of stabilization necessary for the helical configuration in acidic solution. At the other extreme of pH the neutral lysine residues cannot interact in a pairwise manner to provide this extra increment needed to overcome the repulsive effects of the charged glutamate ions; hence the helix is not stable here. Moreover, to the extent that the two amino acid residues are not in random sequence there will be a preference toward clustering of like residues which will diminish helix stability in the pH range 5 to 10.

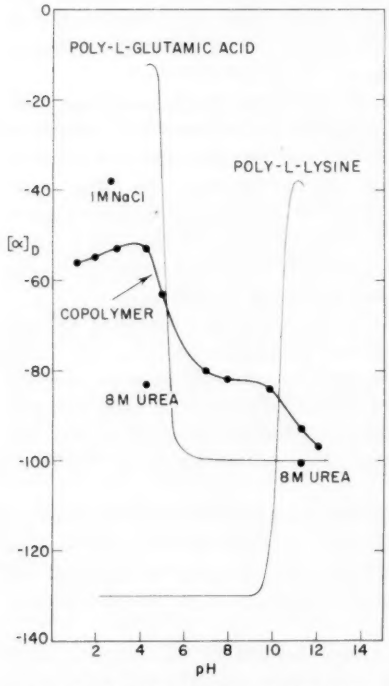

FIG. 1.—Specific rotation of copoly-L-lysine-L-glutamic acid (1:1) as a function of pH in 0.2 *M* NaCl. The effect of 1 *M* NaCl and 8 *M* urea is also shown. The corresponding behavior of poly-L-glutamic acid and poly-L-lysine is shown for comparison.

The helical content of the polypeptide is quite dependent on ionic strength in some regions of pH and disappears altogether in the absence of salt. The increase in optical rotation upon going to 1 *M* NaCl at pH 3 is shown in Figure 1. These effects demonstrate that the electrostatic interaction of the charged groups is critically important, and its attenuation by added electrolyte is necessary for even limited helix formation.

The effect of 8 *M* urea, a common protein denaturing agent, is to diminish, but not necessarily (see Fig. 1 at pH 4.2) eliminate, the helical structure. However, at pH 7 the helical configuration does not exist in 8 *M* urea.

The remaining equilibrium property of the copolypeptide to be reported is its equilibrium titration curve. Initial attempts to determine the titration curve were frustrated by significant downward drifts of pH, particularly above pH 8. These drifts were accompanied by decreases in specific rotation as well. Fortunately, it was found that this behavior was eliminated after exposure to pH 13.

By using a stock solution of the copolypeptide that had been exposed for several hours to pH 13 the titration curve shown in Figure 2 was obtained. The individual points were the result of titrating aliquots of the stock solution. The pH value obtained in each case was stable with time. The specific rotation of each solution was

also measured, and these results are included in Figure 2. The small differences between these specific rotations and those in Figure 1 are due either to differences in the samples used or to failure to attain complete equilibrium in the former case. The apparent pK's obtained from the titration curve are 4.5 and 10.5, in good agreement with the values generally assigned to glutamic acid and lysine in peptides and proteins containing these residues.

Apparent Metastable States.—Solutions of the copolymers that had not been exposed to alkaline pH exhibited to varying degrees two types of behavior, aside from those mentioned above, that seemed to merit a little further investigation. The first of these was the precipitation that occurred upon the addition of alkali. The

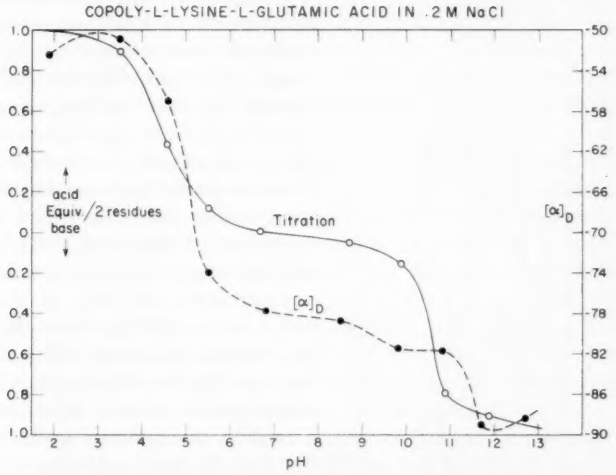

FIG. 2.—Titration (*full line*) and specific rotation (*dashed line*) of copoly-L-lysine-L-glutamic acid as a function of pH in 0.15 *M* NaCl. Measurements made on solution reneutralized from pH 13 to insure equilibrium.

second was the maintenance of the specific rotation characteristic of acidic solutions, i.e., $\sim -50°$, all the way up to pH 9 or 10 under some conditions. When this was displayed, the solutions tended to be cloudy, requiring centrifuging prior to making observations.

The first of these, the temporary precipitation upon the addition of alkali, was not very troublesome because the precipitate always redissolved on standing for a few hours. Since the fraction of material precipitated increased sharply with concentration and since this behavior was reminiscent of the alkaline denaturation of proteins that has been ascribed to the formation of the β-form—that is, the intermolecularly hydrogen-bonded form—we attempted to find out whether the precipitated material did indeed have the β-configuration.

Fortunately, the carbonyl amide I band characteristic of the β-form had just been tentatively identified in D_2O solution in a study of poly-L-lysine.[6] It occurs at 1610 cm.$^{-1}$ and is well separated from the neighboring band at 1640 cm.$^{-1}$ Infrared spectra were taken in D_2O solution immediately following addition of alkali and a few hours later when the precipitate had redissolved. The resulting spectra

are shown in Figure 3. It is seen that the 1610 cm.$^{-1}$ band is predominant in the initial spectra and has vanished in the final spectra. Subject to final proof that the 1610 cm.$^{-1}$ band has been properly identified, the origin of the precipitate and a corresponding cloudiness that develops in more dilute solutions are due to the formation of the β-configuration. This is a metastable state and eventually goes over to the randomly coiled, dissolved polypeptide chains. However, if more uncharged groups were present, it is very probable that the β-form would be stable and that the correspondence with the alkali denaturation of proteins would be complete.

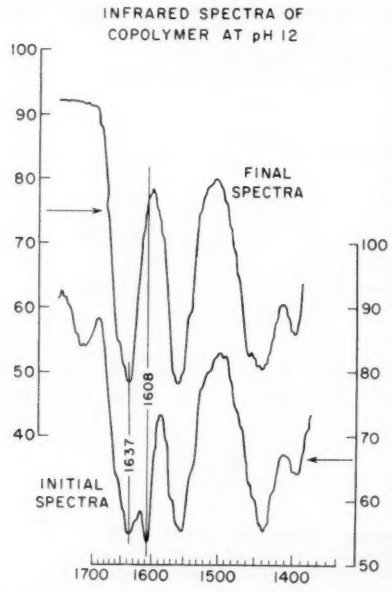

FIG. 3.—Infrared spectra of copoly-L-lysine-L-glutamic acid in D_2O immediately following adjustment to pH 12 and five hours later. The disappearance of the 1,610 cm.$^{-1}$ corresponds to the disappearance of the β-configuration and the resolution of the precipitate that had initially formed. Polypeptide concentration: 3 gm/100 cc. All thickness 0.05 mm.

The other type of behavior associated with a metastable state first appeared in the very long time required for the pH and specific rotation to attain stable values after adjustment of pH to alkaline values. A more extensive investigation showed that more striking aspects of this behavior could be observed in samples of the copolypeptide that had been isolated from the glacial acetic acid by lyphilizing the acid dialyzate. In this form the most extensively studied sample (KI-506) would only redissolve in water or strong alkali, not in saline solutions. If dissolved in water, salt could then be added without precipitation occurring. The acidic saline solution which results from this procedure has a specific rotation equal to that reported in the previous section, $\sim -50°$ for 0.15 M NaCl. However, when the pH is increased to 6 or 7, this rotation is maintained essentially unchanged for days. This contrasts to the fall to about $-80°$ observed under equilibrium conditions. The value of $\sim -50°$ is maintained up to pH values of 9 and 10 but falls very slowly. At pH 12 and 13 it falls rapidly to the equilibrium value, and, upon reneutralization, the value of $-80°$ is obtained. Rotatory dispersion measurements at pH 7 indicated 50 per cent helix and thereby confirmed that the acid-stable configuration was being preserved in a metastable state. Other samples showed these same features but to smaller degrees.[13]

The behavior described here is eliminated by exposure to pH 13 for 24 hours or more. That is, when an alkali-treated solution is dialyzed against 0.001 M HCl and then lyophilized, the sample dissolves directly in neutral saline and, upon stepwise addition of alkali, yields stable pH and specific rotation values. The interactions which give rise to this metastable state have not yet been established, although it is clear that the pH 13 treatment does remove a 1725 cm.$^{-1}$ band from the

infrared spectrum in D_2O and releases glutamic groups for titration. This evidence is compatible with either hydrogen-bonded pairing of glutamic acid groups or the existence of some methyl ester groups, formed while in methanol solution, which would influence the helix stability in some manner as yet unknown. It appears likely that the existence of some methyl ester groups and their subsequent alkaline hydrolysis is the more likely explanation of the behavior described in this section as well as the turbidity development and pH drifts mentioned earlier. If the methanol step is avoided such effects are not found nor has it been possible to reintroduce them into alkaline-exposed copolymers without going through the methanol solution step. Such groups would provide an explanation of the temporary stability of the β-form since they would decrease solubility and the gradual resolution of the copolymer would be due to their gradual hydrolysis at pH 12. The way in which such ester groups would account for the preservation of considerable amounts of helical configuration in neutral and mildly alkaline solution is not so clear, but it may lie in their ability, through their hydrophobic character, to exclude water from the core of the helix.

Behavior in Organic Solvents.—It has already been observed that proteins seldom take up their maximal helical configuration in aqueous solution but that some organic solvents do make this possible.[1, 2] Although ethylene dichloride was originally employed for this purpose, we have found 2-chloroethanol to be more useful because it is a solvent for a wider range of proteins. In this solvent, the copoly-L-lysine-L-glutamic acid is found to be completely helical, as expected. Its specific rotation, $[\alpha]_D$, is +12. Its rotatory dispersion constants are: $b_0 = -636$ and $a_0^H = 871$. These constants are intermediate in value between the corresponding constants for poly-L-glutamic acid and poly-L-lysine. In the infrared spectrum the carbonyl amide I band is very sharp and located at 1655 cm.$^{-1}$.

Much more strongly hydrogen-bonding organic solvents have been found to solvate polypeptides and proteins completely, thereby causing their configuration to be that of structureless randomly coiled chains.[1, 2] Trifluoroacetic acid is such a solvent, and in it the copolymer displays the expected, randomly coiled configuration. The value of $[\alpha]_D$ is $-94°$ and b_0 is zero. Trifluoroacetic acid has the very useful property of being partially transparent in the region of the carbonyl amide I band. The frequency of this band in this solvent is found to be 1635 cm.$^{-1}$. This is sufficiently removed from the frequency characteristic of the helical configuration to permit an accurate estimate of the helical content to be made from the relative intensities of these two bands. The development and application of this method are being reported elsewhere.[15]

Having found that the two principal configurations of the polypeptide chain exist in pure form, one in chloroethanol and the other in trifluoroacetic acid, it is of interest to see how the transition between these two forms takes place as the solvent composition of the mixture of these two solvents is varied. It is already known that pure polypeptides show a very sharp transition in such an experiment.[16] With this copolymer, made of two residues that appear to make different contributions to helix stability, it may be expected that the transition is spread out over a broader range of solvent composition. This is seen to be the case in Figure 4, where the specific rotation is plotted as a function of solvent composition. The ordinate is proportional to helical content. The same behavior is obtained if the helical con-

tent derived from infrared spectra, as indicated above, is plotted against solvent composition. Thus the expected heterogeneity of helix stability is confirmed. It is interesting to compare this result with a typical protein, whose display of the helix-coil transition is not blocked by cystine cross-links. The result of similar measurements with such a protein, ovalbumin, is included in Figure 4. It is seen that the transition occurs at a little lower trifluoroacetic acid concentration and is essentially parallel to the copolymer. From this, one concludes that the range of heterogeneity of helix stability is much the same in the protein and in the copoly-L-lysine-L-glutamic acid.

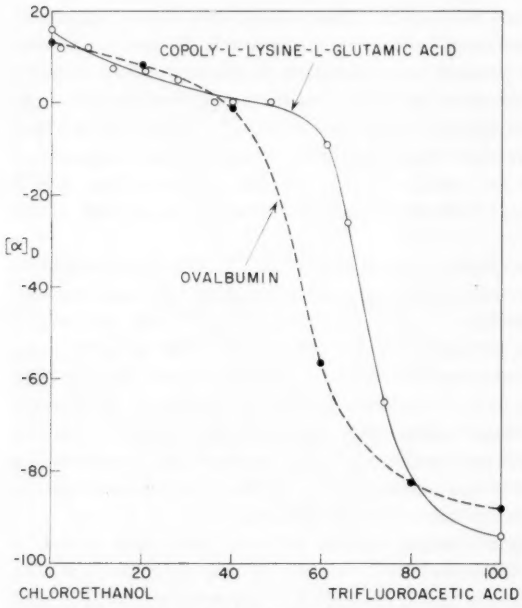

FIG. 4.—The helix-coil transition for copoly-L-lysine-L-glutamic acid and ovalbumin. The specific rotation is plotted as a function of the per cent of trifluoroacetic acid in the chloroethanol-trifluoroacetic solvent system.

Summary.—Equimolar copolymers of L-lysine and L-glutamic acid with molecular weights of about 40,000 were prepared and have been found to exhibit the α-helical configuration to the extent of 15 per cent in neutral 0.15 *M* NaCl. The amount increases to 50 per cent at pH 4 and vanishes at pH 12 or upon removal of the NaCl. This behavior is consistent with the previous observation that under comparable conditions the poly-L-lysine helix is weaker than the poly-L-glutamic helix.[6]

Two apparent metastable states have been observed in copolymers that have not been exposed to alkaline pH: (1) Rapid addition of alkali produces a precipitate which is shown to be the β configuration. It redissolves in a few hours. (2) Initially prepared samples lyophilized from acid solution exhibit in varying degrees partial solubility in water, downward drifts in pH, and specific rotation and hysteresis in alkaline titration. In extreme cases this form maintains the helical content characteristic of acid solutions (50 per cent) all the way up to pH 10. This behavior appears to be due to the blocking of some glutamic acid residues in such a way as to resist titration until the pH is raised beyond 9 or 10. These features disappear after the sample is exposed to pH 13, and thereafter only equilibrium properties are observed. Following such treatment, the expected titration curve is obtained, and in electrophoresis the boundary is sharp and reverses its direction of migration at pH 7.

The addition of urea causes denaturation similar to proteins. In chloroethanol the polypeptide is completely helical. Upon the addition of trifluoroacetic acid, a transition to the randomly coiled form takes place in a manner very similar to proteins such as ovalbumin. This shows that the copolymer has a distribution of more and less stable helix regions comparable to proteins. Furthermore, this solvent system permits the determination of helix content by infrared spectra: for the copolymer and ovalbumin these agree with the results from rotatory dispersion.

We are most appreciative of the aid given us by J. Applequist and R. Salovey and of the helpful advice of Drs. E. Katchalski and E. R. Blout.

* This investigation was supported by the Office of Naval Research (N5 ori-07654) and the National Science Foundation (G-4089).

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[14] Evidence for this was found in the titration and electrophoretic behavior of sample KI 506 before and after alkaline treatment. Before such treatment the equivalents of alkali required to bring a sample from pH 7 to pH 12 was only 85 per cent of that required after exposure to pH 13. Moreover, the direction of migration at pH 7.6 was reversed by alkaline exposure. These observations point to the release of glutamate groups capable of normal titration as the essential effect produced by the alkaline treatment.

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THE PROPERTIES OF SONIC FRAGMENTS OF DEOXYRIBOSE NUCLEIC ACID

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In the molecular characterization of a polymeric substance it is customary to fractionate it into a series of fractions having different molecular weights and then to measure one or more physical constants of the fractions. If the measurements consist of intrinsic viscosity and molecular weight, the degree of coiling of the polymer chain can be assessed, and a calibration is provided for subsequent determinations of molecular weights on other samples. If the measurements also include the sedimentation constants, an alternative calibration of molecular weight is provided, and, in addition, it becomes possible to estimate the molecular-weight distributions from the distributions of sedimentation constants that may be obtained on other samples of the substance.

It is obviously desirable that this kind of information be available for such important substances as deoxyribose nucleic acids (DNA), but it has not been forthcoming, largely because attempts at fractionation of DNA have not yielded fractions covering an adequate range in molecular weights.

In addition to these reasons for having available homologous samples of DNA covering a wide range of molecular weight, there was another. The ability to prepare such a series of samples would make possible the investigation of the dependence of bacterial transformation on the molecular weight of the DNA causing the transformation; this in turn should help in elucidating the molecular basis of this phenomenon.

In these circumstances we undertook an examination of the effects of sonic waves on DNA solutions, in the hope that this would provide a means of preparing a series of samples having decreasing molecular weight but being otherwise undamaged. Some observations made by Laland, Overend, and Stacey,[1] indicating that the viscosity of DNA solutions did fall with exposure to sonic waves, were encouraging because they also recorded that the optical density at 260 mμ was unchanged by the treatment. In view of current knowledge on the denaturation of DNA, this constancy of the optical density demonstrated that the fall in viscosity must have been primarily due to molecular-weight degradation and not to the molecular contraction that accompanies denaturation.

Some of the results of this investigation have been briefly summarized elsewhere.[2] The results reported here have led to an electron-microscopic investigation of sonically produced DNA fragments and to their use in the study of bacterial transformation. This work is being published concurrently.[3, 4]

The Preparation of Sonic Fragments.—The DNA used was prepared in this laboratory from calf thymus, using the B method of Dr. Norman Simmons.[5] Care was taken not to expose the sample to water but only to saline solutions; the successive resolutions were done in 0.1 *M* NaCl. The sample was designated as SB-12A. Light-scattering measurements indicated a molecular weight of 7,400,000

and a radius of gyration of 2500 A. The intrinsic viscosity in 0.2 *M* NaCl, extrapolated to zero gradient, was 69 in units of 100 cc/gm.

Approximately 40-cc. aliquots of about 0.1 gm/100 cc solutions of this DNA in 0.2 *M* NaCl were exposed by placing them directly in the steel cup of a Raytheon magnetostriction, 9-kilocycle, sonic generator operated at about 160 volts. Cooling water was circulated through the outer jacket of the cup during irradiation.

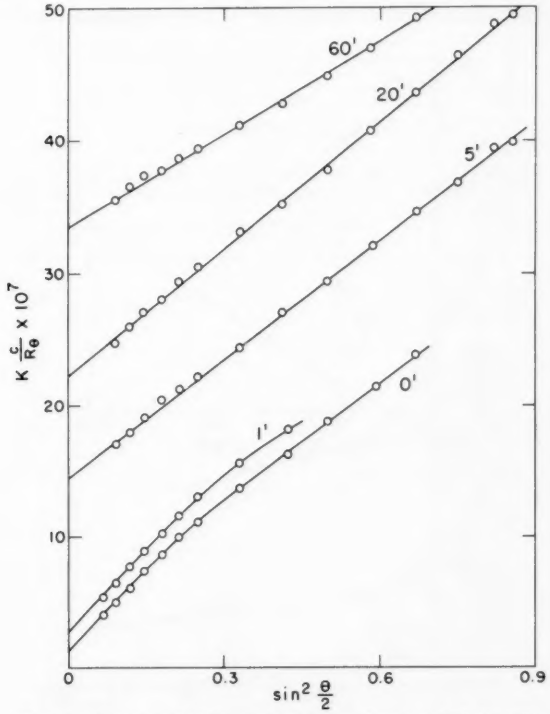

FIG. 1.—Reciprocal light-scattering envelopes at infinite dilution for the undegraded DNA and four sonicates. The numbers on each envelope refer to the minutes of exposure to sonic waves. (dn/dc = 0.188, λ = 4360 A, c = concentration in units of gm/dl).

Nitrogen was bubbled through the solutions immediately prior to irradiation. To provide enough of each sample, three successive aliquots of about 40 cc. were exposed in succession for the same periods of time and then combined. Four such samples were prepared: they corresponded to exposures of 1, 5, 20, and 60 minutes. Such samples are designated "sonicates."

Solutions of DNA treated in this manner showed no change in pH (less than 0.1 unit) or optical density at 260 mμ. Since the constancy of optical density could be established with an accuracy of only 1 per cent and since at this ionic strength a change of only 12 per cent is expected for maximum denaturation, a more sensitive means of detecting damage to the internal structure of the fragments was employed.

This was the determination of the specific viscosity as a function of the temperature to which aliquots of the solution had been heated for $^1/_2$ hour. The control and the 1-minute and 5-minute sonicates showed no perceptible fall in viscosity until 88° C., and half of the ultimate decrease in viscosity was reached at 91°–93°. However, for the 20-minute and 60-minute sonicates the corresponding temperatures were 83° and 88°, respectively. This effect is relatively small and suggests that denaturation had been negligible. By comparison with some studies made on the effect of single-chain scission induced by DNAse treatment,[6] we would estimate

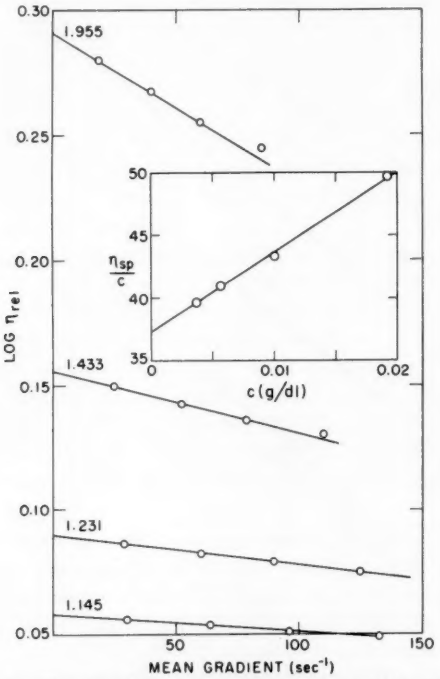

FIG. 2.—The viscosity data for the 1-minute sonicate of DNA. The larger graph shows the log of the relative viscosity as a function of mean gradient. The inset shows the reduced specific viscosity obtained from the intercepts of the larger graph.

that in the 60-minute sonicate no more than one single-chain break per several thousand residues had been made. Such occasional single-chain scissions do not appear to alter the chain configuration until the denaturation temperature is approached. Consequently, we conclude that only a negligible amount of single-chain scission had been produced in the preparation of the sonicates,[7] that no denaturation had occurred, and that the sonicates were homologous with the original DNA.

These conclusions were borne out by the direct electron-microscopic examination that was made of DNA sonicates.[8] Even the 60-minute sonicate showed no signs of denaturation. Occasional kinks in the electron microscope may have been due

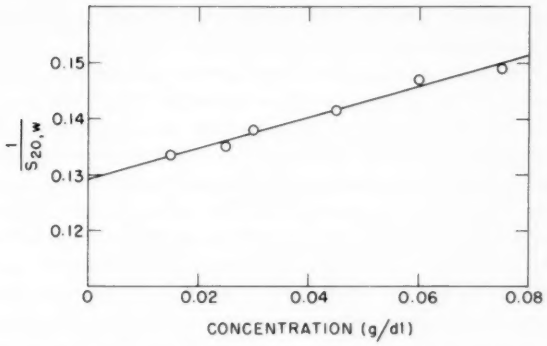

FIG. 3.—Reciprocal sedimentation constants as a function of concentration for the 20-minute sonicate. The intercept yields $s^{\circ}_{20,w} = 7.75\ S$.

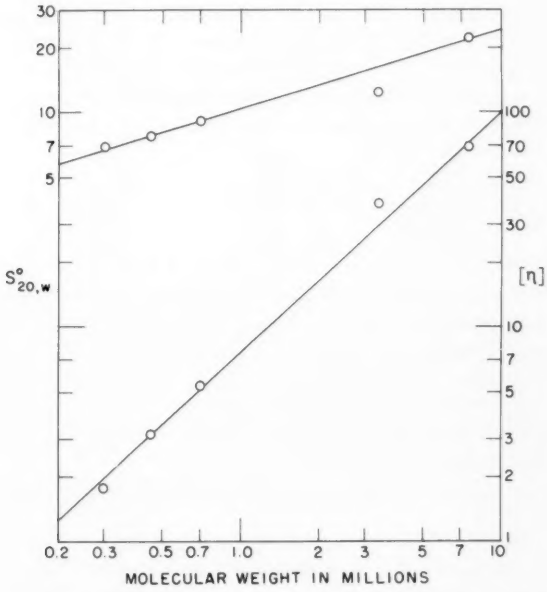

FIG. 4.—A log-log plot of sedimentation constant against molecular weight and intrinsic viscosity against molecular weight for DNA.

to distortion of the molecules at the point of single-chain scissions. In addition to these observations, the electron-microscope investigation provided quantitative evidence on the molecular-weight distribution of the sonicates: it was found to be very close to the most probable distribution, that is, the distribution that results from random double-chain scission. This means that the sonicates had approximately the molecular-weight distribution ascribed to the undegraded DNA.[2]

The Characterization of the Sonic Fragments.—Light-scattering envelopes down to 30° specific viscosities as a function of gradient (in the range of 30–150 sec.$^{-1}$) and sedimentation constants using Schlieren optics and 30-mm path-length cells were determined as a function of concentration on four different sonicates and on the control DNA. The details of the experimental procedures involved have been presented elsewhere.[5, 8] Some of the experimental results are assembled in Figures 1, 2, and 3. In Figure 1 the zero concentration and reciprocal light-scattering plots are shown for each sample. The intercept of these is equal to the reciprocal of the weight average molecular weight,[9] and from the slope the radius of gyration can be determined. In Figure 2 the logarithm of the relative viscosity as a function of the gradient is shown for four different concentrations of the 1-minute sonicate. The reduced specific viscosities obtained from the zero-gradient relative viscosities are plotted in the inset and extrapolate to a value of 37.3, the intrinsic viscosity for this sample. In Figure 3 the reciprocal of the sedimentation constants for the 20-minute sonicate are plotted against concentration. A linear extrapolation yields a value of 7.75 for $s^0_{20,w}$ in units of Svedbergs. These data are typical of those obtained for the other samples. The results are summarized in Table 1.

TABLE 1

MOLECULAR CONSTANTS FOR SONIC FRAGMENTS OF DNA

Time of Irradiation (Min.)	Molecular Weight	$s^0_{20,w}$	$[\eta]$	Radius of Gyration, (A)
0	7,400,000	22.0	69	2500
1	3,400,000	12.2	37.3	1700
5	700,000	9.0	5.35	650
20	450,000	7.75	3.12	540
60	300,000	6.9	1.77	380

Discussion.—The nature of the dependence of sedimentation constant and intrinsic viscosity on molecular weight can be seen in Figure 4, where these data are plotted on log-log co-ordinates. Such plots are essentially linear both for flexible, highly coiled polymers and for rod-shaped macromolecules of constant diameter. Since the behavior of DNA is intermediate between these two cases, it is not unexpected that these plots would also display linearity. Within the probable experimental error, this is seen to be the case.[10]

The lines drawn in Figure 4 are represented by the following expressions:

$$s^0 = 0.063\ M^{0.37},$$
$$[\eta] = 1.45 \times 10^{-6}\ M^{1.12}.$$

The exponent in the viscosity relation is somewhat greater than 1.0, the upper limit for randomly coiled, solvent immobilizing polymers, and it is substantially less than the upper limit of 1.70 for homologous rods of constant diameter. This behavior is not unexpected in view of the highly extended, but slightly flexible and convoluted, configuration associated with DNA.[2, 8]

Further support for this same view comes from examining the molecular-weight dependence of the radius of gyration. For rods the radius of gyration, ρ_g, is proportional to the length. If this rodlike model fitted DNA, one would expect the radius of gyration to be proportional to the length and hence to the molecular weight.

However, the ratio ρ_g/M increases continuously from 3.3 to 12.7 ($\times 10^{-12}$) for the samples listed in Table 1. This shows that, with increasing molecular weight, the molecular size, as measured by the radius of gyration, does not increase as rapidly as the molecular weight. This behavior is more compatible with random-coiled configurations. For random coils the radius of gyration increases only as the 0.5 power of molecular weight when molecular swelling by the solvent is negligible, as it is in this case. The ratio of the radius of gyration to the square root of the molecular weight for the samples is found to be approximately constant, that is, it equals 8.3 ± 1.3 ($\times 10^{-9}$) without any observable trend in molecular weight. Consequently, these observations offer strong support to the configuration already ascribed to DNA molecules in solution.[2, 8]

The theoretically derived dependence of the sedimentation constant on the molecular weight for rods of constant diameter is $M^{0.1}$, and for randomly coiled molecules it is in the range of $M^{0.33}$–$M^{0.50}$. However, the first measurements to become available for a series of homologous rods[11, 12] show a dependence of about $M^{0.20}$. Under these circumstances the value observed for the sonic fragments does not appear to be too large.

The results expressed in the two equations above can be put in another framework of references by relating them to the Scheraga-Mandelkern equation.[13] This relation results from eliminating the frictional coefficient between expressions for s^0 and $[\eta]$ that had been derived for ellipsoids of revolution; it has the following form:

$$\beta = \frac{s^0[\eta]^{1/3}}{M^{2/3}} \frac{\eta_0 N}{(1 - \bar{v}\rho)},$$

where β varies from 2.12 to 3.60×10^6 when the axial ratio of the ellipsoid varies from 1 to very high values ($\sim$300). If the expressions obtained in this study are substituted for s^0 and $[\eta]$, it is found that the value of β increases with molecular weight over the mid-part of the range theoretically available to it. That is, at a molecular weight of 300,000, $\beta = 2.56$; at 3,000,000, $\beta = 3.05$; at 8,000,000, $\beta = 3.29$; at 30,000,000, $\beta = 3.55$. From this, one can conclude that the DNA and its sonicates behave hydrodynamically like ellipsoids of revolution whose axial ratios increase with the molecular weight. Of course, no literal meaning can be attached to the values of the axial ratios that correspond to the β values.

Summary.—By exposing DNA solutions to 9-kilocycle sonic waves for varying times in the absence of oxygen, the molecules were degraded through double-chain scission, producing fragments having the base-paired helical structure intact. In this way homologous samples of DNA were produced that covered a 25 fold range in molecular weight. With these, the molecular-weight dependence of the sedimentation constant, the intrinsic viscosity, and the radius of gyration were determined. The results are summarized by the following relations:

$$s^0_{20,w} = 0.063\, M_w^{0.37},$$
$$[\eta] = 1.45 \times 10^{-6}\, M_w^{1.12},$$
$$\rho_g = 8.3 \times 10^{-9}\, M_w^{0.5}.$$

The first of these relations can serve as a basis for estimating the molecular-weight distribution of DNA from sedimentation distributions obtained with ultraviolet

optics. By combining the first two relations, it is found that the DNA samples exhibit acceptable β-values in the Scheraga-Mandelkern equation and that these increase with molecular weight. The last relation supports other evidence that DNA molecules, even though they are highly extended, are not rodlike but gently coiled in a random fashion in solution.

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*LABELING OF PROTEINS BY ISOTOPIC AMINO ACID DERIVATIVES**

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Previous publications[1–5] from this laboratory have described the catalysis, by intracellular proteolytic enzymes, of transamidation reactions of the type RCO—NH_2 + $NH_2X \rightleftharpoons$ RCO—NHX + NH_3, where the first reactant is a suitable substrate (e.g., glycyl-L-tyrosinamide for cathepsin C) and the second reactant ("replacement agent") is a peptide (e.g., L-leucylglycine) or hydroxylamine. It has been postulated[6] that in such reactions the substrate combines with the enzyme to form an intermediate acyl-enzyme (RCO—E), which then reacts either with water, resulting in hydrolysis, or with NH_2X, resulting in transamidation.[7] The experiments reported in the present communication indicate that insulin and the proteins of rat liver mitochondria can also serve as replacement agents in enzyme-catalyzed transamidation reactions. In these experiments, C^{14}-labeled tyrosine derivatives were employed, and their "incorporation" into the above proteins was measured. Because of the suggestion that aminoacyladenylates are intermediates in the biosynthesis of proteins,[8–11] such C^{14}-labeled compounds also were synthesized, and their "incorporation" into the mitochondrial proteins was examined.

Labeling of Insulin by Isotopic Glycyltyrosinamide.—Glycyl-L-tyrosinamide (GTA) is a synthetic substrate for the intracellular proteinase cathepsin C,[12] which has been partially purified from beef spleen and which specifically catalyzes hydrolytic and replacement reactions involving dipeptide amides or dipeptide esters.[4, 13] C^{14}-labeled GTA acetate was prepared in the manner described previously[4]; uniformly labeled L-tyrosine (18,000 cpm/μmole) was used as starting material. In a typical experiment with insulin as the replacement agent, the incubation mixture (2 ml.) contained: 5 mg. C^{14}-GTA (16.5 μmoles; 297,000 cpm); 60 mg. insulin (Lilly); cathepsin C (0.2 unit; 0.036 mg. protein); cysteine (10 μmoles). The pH was adjusted to 7.0 with NaOH, and the mixture was incubated for 90 minutes at 38° C. During this time, the GTA had been completely deamidated, as shown by parallel measurement of NH_4^+ formation. The incubation mixture was chilled, and 0.66 ml. of 20 per cent trichloroacetic acid was added. The resulting precipitate was centrifuged, washed three times with cold 5 per cent trichloroacetic acid, and dissolved in 2 ml. of cold 0.1 *N* NaOH. Unlabeled glycyl-L-tyrosine (5 mg. in 0.5 ml.) was added, and the dissolved protein was reprecipitated by the addition of 1.8 ml. of 20 per cent trichloroacetic acid, centrifuged, and washed three times with cold 5 per cent trichloroacetic acid. Upon the addition of 2 ml. of ethanol to the centrifuged precipitate, the protein dissolved and was reprecipitated with 4 ml. of ether. The resulting material was washed with ether, dried, and its radioactivity was determined in a gas-flow counter; about 1 mg. was placed on 1-cm.^{2} circles for counting. From the above experiment, 41.6 mg. of protein were obtained; its specific radioactivity was 200 cpm/mg, corrected for self-absorption.

Control experiments in which the enzyme had been omitted or in which GTA had been replaced by labeled glycyl-L-tyrosine (GT) of the same specific radioactivity gave protein samples of negligible C^{14}-content (0 and 5 cpm/mg, respec-

tively). This indicates that the radioactivity "incorporated" into the insulin in the above experiment was not caused by adsorption of unchanged GTA or of GT formed by enzymic hydrolysis. To determine whether the radioactivity was a consequence of the adsorption of the glycyltyrosyl polymer formed by cathepsin C from GTA,[3] control experiments were conducted in which insulin (either untreated or previously incubated with cathepsin C in the absence of GTA) was added to an incubation mixture from which insulin had been omitted at the start. Trichloroacetic acid was added immediately thereafter; the protein sample obtained in the manner described above had a C^{14}-content of 3 cpm/mg. Furthermore, C^{14}-labeled polymer (4,290 cpm/mg) was isolated from an incubation mixture containing isotopic GTA and cathepsin C at pH 7.5 (no insulin present). Upon mixing 1.2 mg. of the labeled polymer with 20 mg. of insulin, followed by the reprecipitation procedure outlined above, a protein sample having 8 cpm/mg was obtained. In addition, mixtures of the polymer and insulin could be separated by paper chromatography, with 2-butanol saturated with 1 per cent acetic acid as the solvent; under the conditions employed in these chromatographic runs, the polymer remains at the origin, and the insulin moves with an R_F of about 0.15. When the labeled insulin was subjected to such chromatographic treatment, all the radioactivity was associated with the insulin spot. It appears unlikely, therefore, that the protein labeled by incubation in the presence of isotopic GTA contained appreciable amounts of adsorbed polymer.

It may be added that, under the conditions employed, the amount of cathepsin C present in the complete incubation mixture was insufficient to cause a detectable hydrolytic cleavage of insulin, as measured by means of a Radiometer autotitrator. Also, separate experiments indicated that the rate of enzymic deamidation of GTA was not appreciably changed by the presence of insulin in the incubation mixture.

To establish whether the glycyltyrosyl residue of GTA had become attached to one of the amino groups of insulin, the labeled protein was subjected to papain digestion[14] for 24 hours, and the incubation mixture was treated with charcoal to adsorb aromatic compounds. Most of the radioactivity could be adsorbed on charcoal, from which it was eluted with ethanol. Paper chromatography of the ethanol extract, with *n*-butanol–acetic acid–water (3:1:1) as the solvent, gave three radioactive ninhydrin-positive components having R_F values of 0.52, 0.70, and 0.86. The component of R_F 0.86 had the greatest C^{14}-content and was investigated further. It migrated as a single spot in several other solvents: in trimethylcarbinol-methyl ethyl ketone-ammonia-water (10:10:3:5) its R_F was 0.43; in 2,6-lutidine-water (65:35) its R_F was 0.13; in pyridine-methanol-water (1:40:10) its R_F was 0.41; and in 2-butanol–formic acid–water (75:15:10) its R_F was 0.18. On acid hydrolysis (6 *N* HCl, 24 hours, 110° C.), this apparently homogeneous component disappeared and gave rise to 5 ninhydrin-positive components whose R_F values (in several solvents) were identical to those of authentic samples of glutamic acid, glycine, tyrosine, valine, and isoleucine (or leucine). Only the spot corresponding to tyrosine was radioactive. Since the *N*-terminal sequence of the *A*-chain of insulin is glycyl-isoleucyl-valyl-glutamyl-,[15] and since papain is known to hydrolyze readily peptide bonds involving a glutamyl residue,[16, 17] it may be inferred that papain had released from the labeled protein a fragment of the *A*-chain to which one or more glycyltyrosyl residues had become attached by peptide

linkage. It would appear, therefore, that the α-amino group of the *N*-terminal glycyl residue of insulin had participated in a cathepsin C–catalyzed transamidation reaction in which a C^{14}-labeled glycyltyrosyl residue had become "incorporated" into the protein.

Since the papain digest appeared to contain at least two radioactive components other than that discussed above, the possibility exists that, in addition to the *N*-terminal glycyl residue, other constituents of insulin (e.g., the *N*-terminal phenylalanyl residue of the *B*-chain) may have participated in replacement reactions. However, studies with synthetic replacement agents have indicated that, at pH values near 7.5, phenylalanyl peptides tend to be less efficient as replacement agents, and the ϵ-amino group of lysine (also present in the *B*-chain) is ineffective. Further studies on the composition of the two radioactive components of lower R_F may show, therefore, that they represent other fragments of the *N*-terminal sequence of the *A*-chain.

Labeling of Mitochondrial Proteins by Isotopic Tyrosine Derivatives.—The findings summarized above indicate that a proteolytic enzyme normally present in animal tissues can catalyze a transamidation reaction leading to the substitution of an α-amino group of a protein by an activated acyl group. Such reactions may occur in incubation mixtures used in the study of the incorporation of labeled "activated" amino acids into subcellular fragments. For this reason, experiments were undertaken on the incorporation of labeled amino acid residues into the proteins of rat liver mitochondria. The studies of Simpson and his associates[18] have shown that, in the presence of an ATP-generating system, the mitochondrial fraction of rat liver homogenates readily incorporates labeled amino acids into the mitochondrial protein. In the present experiments, an ATP-generating system was omitted, and the labeled compounds were the amides or acyladenylates of L-tyrosine and glycyl-L-tyrosine. Male rats (Sprague-Dawley) weighing about 60 gm. were killed, the livers were ground with 0.25 *M* sucrose in a loose glass homogenizer, and the mitochondrial fraction was separated by differential centrifugation in the manner described by Schneider and Hogeboom.[19] The mitochondria were washed four times with 0.25 *M* sucrose, the mitochondrial pellet being resuspended by homogenization between washings. The incubation mixture (1 ml.) contained: 5 μmoles of labeled compound; the mitochondrial suspension (0.1 ml., from about 0.2 gm. liver), which contained about 3 mg. protein; 50 μmoles tris(hydroxymethyl)aminomethane (pH 7.5); 5 μmoles KCl; 5 μmoles $MgCl_2$. All incubations were done in duplicate. After being shaken for the requisite time at 38° C., trichloroacetic acid was added, and the protein material was washed four times with cold 5 per cent trichloroacetic acid, once with hot (90° C.) trichloroacetic acid, and dissolved in 4 ml. 0.4 *N* NaOH. Unlabeled tyrosine or glycyltyrosine (25 μmoles in 0.5 ml.) was added to wash out adsorbed radioactivity, and the protein was reprecipitated with trichloroacetic acid and washed successively with ethanol, ethanol-ether, and ether.

C^{14}-labeled L-tyrosinamide was prepared from uniformly labeled L-tyrosine (31,000 cpm/μmole) by treatment of the ethyl ester with NH_3 in methanol[20]; m.p., 153°–154° C. When this compound was incubated with mitochondria under the conditions given in the preceding paragraph (total radioactivity in the incubation mixture, 155,000 cpm), the mitochondrial protein obtained became labeled, the extent of isotope "incorporation" increasing with longer periods of incubation.

The specific radioactivity obtained after an incubation period of 30 minutes was 15 cpm/mg; after 60 minutes, 42 cpm/mg; after 90 minutes, 96 cpm/mg. These findings may be compared with the result obtained by Simpson *et al.*[18] on the extent of labeling observed upon incubating mitochondria (from 1 gm. liver) with 0.3 μmole of C^{14}-leucine (500,000 cpm) in the presence of an ATP-generating system and the "pH 5 fraction" described by Hoagland *et al.*[8] After an incubation period of 20 minutes, precipitation of the mitochondrial protein in a manner similar to that described above gave a material having 20 cpm/mg protein. We are indebted to Dr. Simpson for permission to cite this result prior to the publication of the detailed account of his studies.

The fact that the labeling of mitochondrial protein by C^{14}-tyrosinamide involves an enzyme-catalyzed reaction is indicated by the finding that, if the mitochondrial fraction is heated for 3 minutes at 100° C., its ability to become labeled is lost. The protein obtained after 60 minutes incubation with such a heated preparation had 4 cpm/mg; after 90 minutes' incubation, 2 cpm/mg. It has long been known that animal tissues contain enzyme activity toward L-tyrosinamide;[21] in connection with the present studies, the ability of rat liver mitochondria to deamidate tyrosinamide was examined. Under the conditions of the incubation with the isotopic compound, L-tyrosinamide is deamidated to about 50 per cent in about 30 minutes, the reaction being complete at about 100 minutes. This enzymic activity may be attributed tentatively to an aminopeptidase of the type previously found in swine kidney.[21, 22]

Control experiments with C^{14}-labeled L-tyrosine showed that the observed labeling of the mitochondrial protein could not be attributed to adsorption of the amino acid formed by hydrolysis of the amide; it would appear that the method employed for the preparation of the mitochondrial protein for counting was adequate to remove such absorbed tyrosine. Samples of the incubation mixture treated with trichloroacetic acid immediately after mixing ("zero time incubation") gave samples of protein with negligible radioactivity (2 cpm/mg), indicating that essentially all the unchanged tyrosinamide that may have been adsorbed was removed by the precipitation method employed in these experiments.

A sample of the labeled mitochondrial protein obtained from experiments with C^{14}-labeled tyrosinamide was treated with 1-fluoro-2,4-dinitrobenzene at pH 9, and then hydrolyzed with 6 *N* HCl for 12 hours at 110° C. The hydrolyzate was extracted with ether, which readily dissolves dinitrophenylamino acids, including *O,N*-diDNP-tyrosine. It was found that about 90 per cent of the total radioactivity of the acid hydrolyzate was present in the ether extract, suggesting that nearly all of the C^{14}-tyrosine residues bound to the mitochondrial protein were located at *N*-terminal positions. One may tentatively conclude, therefore, that the "incorporation" of the isotopic tyrosyl residue had been effected by an enzyme-catalyzed transamidation reaction analogous to that discussed above in connection with the labeling of insulin by isotopic GTA. This conclusion is supported by the finding that the addition of 20 μmoles of hydroxylamine to the incubation mixture containing C^{14}-tyrosinamide reduces the extent of labeling of the mitochondrial protein in 60 minutes from 42 to 13 cpm/mg. In separate experiments, the enzymic formation of a hydroxamate could be demonstrated colorimetrically. The finding that most of the "incorporated" radioactivity appears to be associated with *N*-terminal

amino acids makes it unlikely that the tyrosyl residue had become attached to the side-chain carbonyl group of aspartic or glutamic acid in transamidation reactions involving asparaginyl or glutaminyl residues of the mitochondrial proteins.[23]

Initial experiments with C^{14}-labeled GTA indicate that its tyrosyl residue is "incorporated" into mitochondrial protein less effectively than is the case with C^{14}-tyrosinamide. The presence of cathepsin C in rat liver mitochondria has been reported,[24] and further studies with labeled GTA are needed to explain the difference in the extent of labeling observed with the two C^{14}-tyrosine derivatives.

Although the labeling of mitochondrial protein by C^{14}-tyrosinamide suggests that mitochondrial proteolytic enzymes can catalyze the transfer of aminoacyl groups to terminal amino acids of the protein, it cannot be concluded at present that α-amides of amino acids play a role in protein metabolism. However, as noted previously,[25] such amides are not so unphysiological as was once thought, and their metabolism merits closer investigation. A more general conclusion to be drawn from the experiment with C^{14}-tyrosinamide is that the demonstration of the labeling of a subcellular protein fraction by an isotopic amino acid cannot be considered to be evidence of protein synthesis, even if it is shown that the labeled amino acid is linked to the protein by a peptide bond. A more stringent criterion is the demonstration that the labeled amino acid appears at an interior position of the peptide chain of a reasonably well-defined single protein; an example of efforts in this direction is the work of Bates *et al.*[26] on the incorporation of labeled valine into the cytochrome c of liver mitochondria.

The work of numerous investigators[8–11] has led to the view that the "activation" of amino acids for protein synthesis involves a reaction with ATP, to form α-aminoacyladenylates. To compare the effectiveness of such compounds in the labeling of mitochondrial protein with that observed in the case of C^{14}-tyrosinamide, the corresponding C^{14}-tyrosyladenylate was synthesized. The synthesis involved the reaction of equimolar proportions of carbobenzoxy-L-tyrosine and adenosine-5′-phosphate in the presence of a slight excess of dicyclohexylcarbodiimide in a solution of tetrahydrofuran-water (1:1) containing 10 per cent pyridine. The carbobenzoxytyrosyladenylate could not be obtained in a state of analytical purity, because of the instability of the compound and since difficulty was encountered in separating it from adenosine-5′-phosphate. A less impure product was obtained by the same method in the reaction of carbobenzoxyglycyl-L-tyrosine with adenosine-5′-phosphate; calculated for $C_{29}H_{32}N_7O_{12}P$ (701.6), N 14.0, P 4.6; found, N 14.0, P 4.5. The tyrosine content (Folin-Ciocalteu method) of the latter product was found to be 26.3 per cent; theory, 25.7 per cent; the molar absorbance at 260 mμ in aqueous solution was 13,700. Both carbobenzoxytyrosyladenylate and carbobenzoxyglycyltyrosyladenylate reacted immediately with aqueous hydroxylamine or aqueous ammonia to form the expected hydroxamate or amide. These adenylates are rapidly hydrolyzed at acidic and alkaline pH values and are most stable in aqueous methanol near pH 7.

Immediately before use for experiments with rat liver mitochondria, the carbobenzoxy compounds were subjected to hydrogenolysis in methanol containing 5 equivalents of acetic acid, with freshly prepared palladium black as the catalyst. The concentration of tyrosyladenylate or of glycyltyrosyladenylate was determined by the hydroxylamine reaction, and 5 μmoles of the acyladenylate were added per

ml. of the incubation mixture. Control experiments showed that the adenosine-5′-phosphate present as an impurity did not affect the outcome of labeling experiments.

When 5 μmoles of a preparation of C^{14}-labeled tyrosyladenylate (12,000 cpm/μmole) were incubated with mitochondria under the conditions described above, the mitochondrial protein became labeled. After 10 minutes' incubation, the protein had 122 cpm/mg; after 30 or 60 minutes, 195 cpm/mg. The "zero time" incubation gave a protein sample having 2 cpm/mg. In the presence of 20 μmoles of hydroxylamine, the isotope incorporation into protein is completely abolished.

In contrast to the result with C^{14}-tyrosinamide, the labeling of mitochondrial protein by C^{14}-tyrosyladenylate cannot be considered to be an enzymic process, since mitochondria that had been heated for 3 minutes at 100° C. were labeled even more effectively. Under strictly comparable conditions, incubation of labeled tyrosyladenylate with heat-treated mitochondria for 30 minutes gave protein samples whose average radioactivity was 1,260 cpm/mg, instead of 195 cpm/mg found with unheated mitochondria. This six-fold increase in labeling is consistent with the view that tyrosyladenylate acted as a chemical acylating agent (like acetic anhydride) and that the denaturation of the mitochondrial proteins caused by the heat treatment made more groups (probably amino groups) available for non-enzymic acylation. Further evidence for such substitution of groups in the mitochondrial protein is provided by the result of treatment, with 1-fluoro-2,4-dinitrobenzene, of protein labeled in the incubation of unheated mitochondria with C^{14}-tyrosyladenylate. As in the experiment with labeled tyrosinamide, the acid hydrolyzate of the DNP-protein contained about 90 per cent of the radioactivity in the form of ether-extractable DNP-compounds, presumably *O,N*-diDNP-tyrosine.

If equimolar amounts (5 μmoles) of C^{14}-labeled tyrosinamide and of unlabeled tyrosyladenylate are incubated with liver mitochondria, the extent of labeling of the mitochondrial protein during a 60-minute incubation period is decreased from 42 to 24 cpm/mg, suggesting that the enzyme-activated tyrosyl residue from tyrosinamide and the reactive tyrosyl residue of tyrosyladenylate are competing for similar groups in the mitochondrial protein. It will be of interest to examine, with subcellular fractions, the competition between the tyrosyl residue of tyrosinamide and "activated" tyrosine formed from the free amino acid in the presence of an ATP-generating system and the "pH 5 fraction."

In view of the above findings, it is not surprising that C^{14}-labeled glycyltyrosyladenylate also causes labeling of the mitochondrial proteins, as do the carbobenzoxy derivatives of the two acyladenylates. For example, after incubation of mitochondria for 60 minutes with 5 μmoles of labeled carbobenzoxy-L-tyrosyladenylate (total radioactivity, 60,000 cpm/ml), the mitochondrial protein contained 196 cpm/mg.

While these experiments were in progress, we learned that Castelfranco, Moldave, and Meister had independently synthesized C^{14}-labeled glycyladenylate and tryptophyladenylate via the carbobenzoxy derivatives and had found that, when such compounds are incubated with the microsomal fraction of rat liver homogenates, the microsomal proteins become labeled. Moreover, when the microsomal fraction is heated before incubation with the labeled compounds, the extent of isotope "incorporation" is markedly increased, suggesting that non-enzymic acylation by

the highly reactive aminoacyladenylates is responsible for the labeling. We are grateful to Dr. Meister for permission to mention his results prior to their publication.

It should be emphasized that the experiments on the non-enzymic labeling of cellular proteins with synthetic aminoacyladenylates cannot be considered to disprove the hypothesis that such compounds are intermediates in the biosynthesis of proteins from amino acids. Obviously, the addition of a relatively large amount of an active acylating agent to a subcellular preparation is not equivalent to the coupled enzymic generation of such a compound and its rapid utilization in subsequent reactions catalyzed by a multi-enzyme system. The results reported in the present communication, however, indicate the need to take into account, in studies on the incorporation of labeled amino acids into cellular fragments, the possibility of non-enzymic acylation by reactive acid anhydrides that may be formed in biological systems.

Summary.—The intracellular proteinase cathepsin C catalyzes a transamidation reaction between C^{14}-labeled glycyl-L-tyrosinamide and insulin, leading to the labeling of the protein by substitution of an *N*-terminal amino acid residue. Similar transamidation reactions appear to be operative in the enzymic labeling of the proteins of rat liver mitochondria upon incubation of mitochondria with C^{14}-labeled tyrosinamide. The mitochondrial proteins may also be acylated by C^{14}-labeled L-tyrosyladenylate or glycyl-L-tyrosyladenylate, as well as by their *N*-carbobenzoxy derivatives; this process appears to be largely non-enzymic in nature, since it is promoted, rather than inhibited, by heat denaturation of the mitochondrial proteins.

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*THE MECHANISM OF CHEMICAL MUTAGENESIS. I. KINETIC STUDIES ON THE ACTION OF TRIETHYLENE MELAMINE (TEM) AND AZASERINE**

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Quantitative studies on the kinetics of chemical mutagenesis in bacteria have not paralleled in number investigations on radiation-induced mutations.[1] The work of Demerec and Hanson[2] on chemically induced mutagenesis in *Escherichia coli* illustrates a fruitful approach to this problem and had its origin in two developments: the discovery of a mutagen of relatively low toxicity—manganous chloride—and the introduction of a simple and efficient assay system for measuring the mutation rate from streptomycin dependence to independence. The main emphasis in the work of the Demerec group,[3–4] however, subsequently shifted from quantitative studies of a particular mutational system to the evaluation of the specificity of several mutagens on a variety of genetic loci.

The present work represents an examination of two other potent mutagens—triethylene melamine (TEM) and azaserine— which are active over wide ranges of concentration and temperature without excessive killing. This study was prompted by an awareness of the advantages of a mutagenic system which would lend itself to quantitative kinetic studies of chemically induced mutational processes and thus provide a tool for deeper insight into the mechanism of events leading to a mutation.

MATERIALS AND METHODS

The streptomycin-dependent (*sd-4*), cysteine-requiring (*cys-2*) strain Sd4-73 of *E. coli* was obtained from Dr. M. Demerec. The stock culture was periodically purified by selection from among a number of single-colony isolates on the basis of a low proportion of spontaneous back-mutants.[5] Nutrient broth (Difco) containing 20 μg/ml of streptomycin was employed for preparing liquid cultures. Three solid media served for enumeration of the total number of colonies and the proportion of respective mutants: Difco nutrient agar with and without 100 μg/ml of streptomycin, and minimal agar (7 gm. K_2HPO_4; 2 gm. KH_2PO_4; 0.5 gm. Na_3 citrate·$5H_2O$; 0.1 gm. $MgSO_4 \cdot 7H_2O$; 1 gm. $(NH_4)_2SO_4$; 2.5 gm. glucose; 20 gm. agar) with 100 μg/ml streptomycin.

In the general procedure, an overnight culture of Sd4-73 was prepared by incubation with forced aeration at 32° C. The cells were centrifuged, washed twice, and finally suspended in distilled water, to which the desired amount of mutagen had been added prior to incubation at a particular temperature for a chosen period of time. After mutagen treatment, the cells were washed free of the mutagen,

whenever necessary, and assayed for survivors and mutants on appropriate media. The number of induced mutants was calculated in relation to the number of surviving cells and corrected by subtracting the corresponding value obtained in a parallel control experiment with the mutagen-free cell suspension. Extensions of this general procedure will be described in the discussion of individual experiments. This procedure was adapted from the method described by Demerec and Hanson, with the elimination of prewashing the cells in 0.3 *M* NaCl. The pronounced stimulation of the mutagenicity of Mn^{++} ions by hypertonic treatment[2] could not be demonstrated for azaserine and TEM (Table 1).

EXPERIMENTAL

Effect of Mutagen Concentration (Fig. 1).—In the initial experiments the mutagen concentration was varied, with the expectation of finding a "plateau" region in the concentration-mutagenicity curve where changes in concentration might have little or no effect on the number of mutants induced—a region which would be best suited to the study of other variables. Such a plateau has been observed for man-

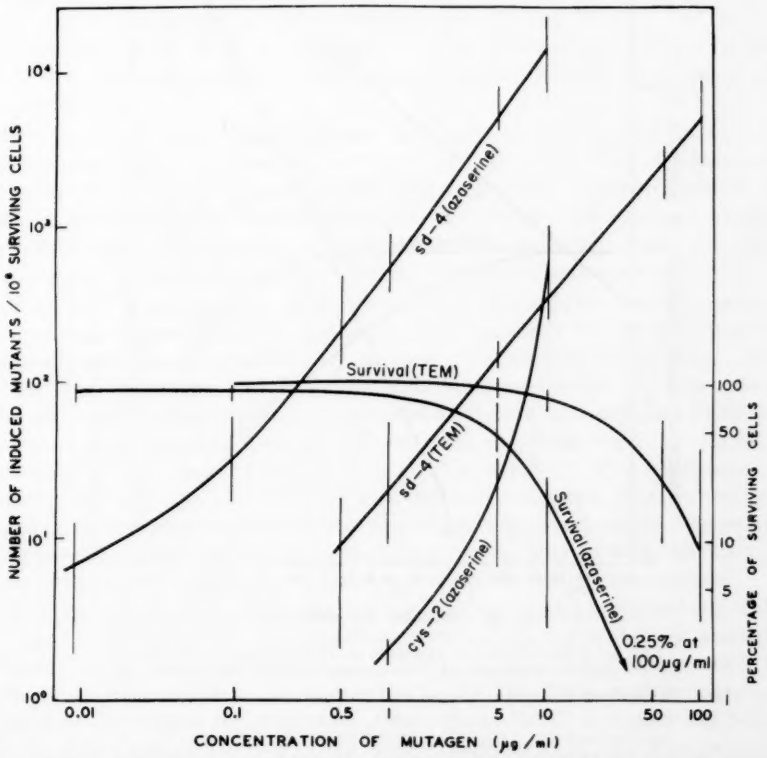

FIG. 1.—Effect of the concentration of TEM and azaserine on the number of induced mutants (*sd-4* and *cys-2* reversions) and on the percentage of survivors (2 hours exposure; 32° C.). The vertical lines denote the spread of pooled experimental results from several experiments. They were omitted in Figs. 2 and 3 to preserve the clarity of the presentation.

TABLE 1

EFFECT OF PRETREATMENT WITH 0.3 *M* NaCl ON NUMBER OF MUTANTS INDUCED BY 2-HOUR TREATMENT WITH TRIETHYLENE MELAMINE (TEM), AZASERINE, AND MANGANOUS CHLORIDE

Mutagen (Conc.)	Cells Pre-washed with 0.3 *M* NaCl	Per Cent Survivors	Induced Mutants per 10^8 Survivors *sd-4*	*cys-2*
TEM.....	−	80.5	330	1.6
(5 μg/ml)	+	72.4	230	0.4
Azaserine...	−	68.6	2,200	66
(5 μg/ml)	+	44.7	3,000	36
$MnCl_2$...	−	100	14	0
(400 μg/ml)	+	89.5	1,100	180

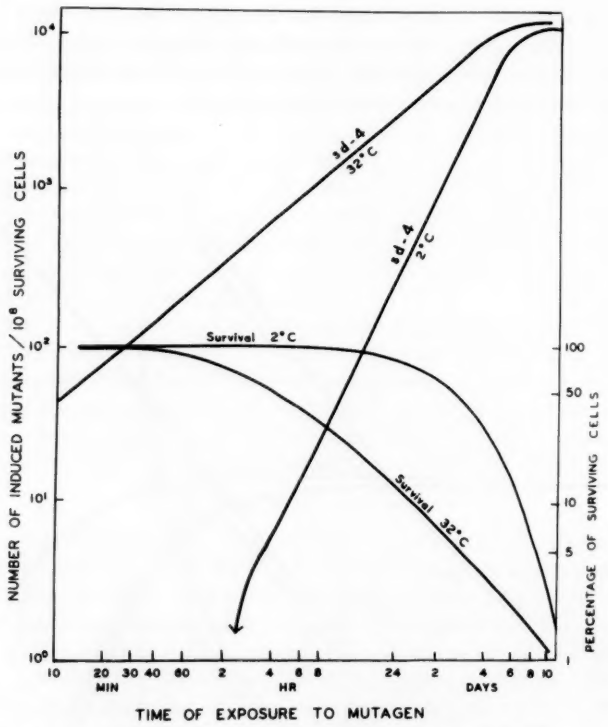
NUMBER OF INDUCED MUTANTS / 10⁸ SURVIVING CELLS
PERCENTAGE OF SURVIVING CELLS
sd-4 32°C
sd-4 2°C
Survival 2°C
Survival 32°C
10 20 30 40 60 2 4 6 8 24 2 4 6 8 10
MIN HR DAYS
TIME OF EXPOSURE TO MUTAGEN

FIG. 2.—Effect of time of exposure to TEM (10 μg/ml) on the number of induced mutants (*sd-4* reversions) and on the percentage of survivors, at 32° and 2° C.

ganous chloride by Demerec and Hanson.[2] In our experiments, cells were exposed to varying concentrations of mutagen at an arbitrarily chosen temperature of 32° C. for 2 hours. The results for TEM and azaserine were as follows: (1) Increasing the concentration of either TEM or azaserine did not produce a mutagenic

response of the type characteristic of manganous chloride;[2] for both mutagens, the relationship between the logarithms of the two variables was nearly linear, with a slope of approximately 1. (2) Over wide concentration ranges in which the mutagens were active (TEM, 1–50 μg/ml; azaserine, 0.01–5 μg/ml), survival was close to 100 per cent, falling off rapidly beyond these limits. (3) Azaserine-induced mutations of *cys-2* to prototrophy paralleled those of *sd-4* to streptomycin independence, although the number of mutants was smaller by a factor of 25–40. The effect of TEM on *cys-2*, under the experimental conditions, was too small for evaluation.

The absence of a "plateau" region necessitated rigid control of the concentration level in subsequent experiments, a condition rather difficult to fulfill with unstable substances. For the same culture, the results were reproducible with a variation of less than ± 25 per cent. In experiments performed on different cultures, the mutagenic response varied within much wider limits, which are indicated by the vertical bars corresponding to each point on the curves (Fig. 1).

Effects of Time and Temperature (Figs. 2 and 3).—Experiments in this series were conducted at two arbitrarily chosen temperatures (32° and 2° C.). The selected concentrations of 10 μg/ml of TEM and 5 μg/ml of azaserine were known from previous experiments to produce low bactericidal effects at sufficiently high mutagenicity (Fig. 1).

With TEM, an almost linear response (log-log scale) was obtained for exposure times ranging from 10 minutes to 3 or 4 days at 32° C., the number of mutants per 10^8 survivors increasing by approximately 150 every hour. After this period, the curve leveled off, and the fraction of mutants per 10^8 survivors remained constant (at $1\text{–}2 \times 10^4$). The number of survivors steadily decreased during the entire period of observation, even after the "plateau" for induced mutants was reached.

In the assay of mutagenicity at low temperatures, the cells were never exposed to a temperature higher than 2° C. during the entire operation, including prewashing, treatment with mutagen, centrifuging, removal of the mutagen, and plating. During the first few hours, no induced mutants were perceptible. In experiments extended over a period of days, it soon became apparent not only that TEM was capable of exerting mutagenicity at low temperatures but also that the maximum number of induced mutants was identical at 2° and 32° C. This indifference to temperature renders invalid the otherwise legitimate objection that, during prolonged treatment, slow multiplication of non-dependent mutants at the expense of a dying parental population may have been involved, by analogy with the interpretation of the origin of streptomycin resistance in quasi-stationary populations.[6]

The shape of the survival curve at 2° C. was indicative of a lower toxicity of the mutagen at this temperature and closely approximated the curve obtained when the cells were stored in distilled water at 2° or 32° C. in the absence of mutagen. In these control experiments an increase in the number of spontaneous mutants was noted, with doubling of the proportion of mutants at 32° C. over an 8-day period and an almost tenfold increase in the same time span at 2° C. The indicated mutagenic effect of cold was also observed by Ryan[7] and Wainright,[8] and can perhaps be better understood through a consideration of the paradoxical results presented in Figure 5 and discussed in the corresponding section of this paper.

The effect of time and temperature on the action of azaserine is depicted in Figure 3. The maximum effect of this mutagen at 32° C. was reached in a period of 10–20 hours, i.e., much earlier than for TEM. At 2° C., the final number of mutants produced by azaserine at a concentration of 5 μg/ml was somewhat lower than at 32° C.

These data extend the results of Demerec and Hanson,[2] who reported a marked decrease in the short-term mutagenicity of $MnCl_2$ at low temperatures but did not study the effect of the mutagen over a prolonged period of time.

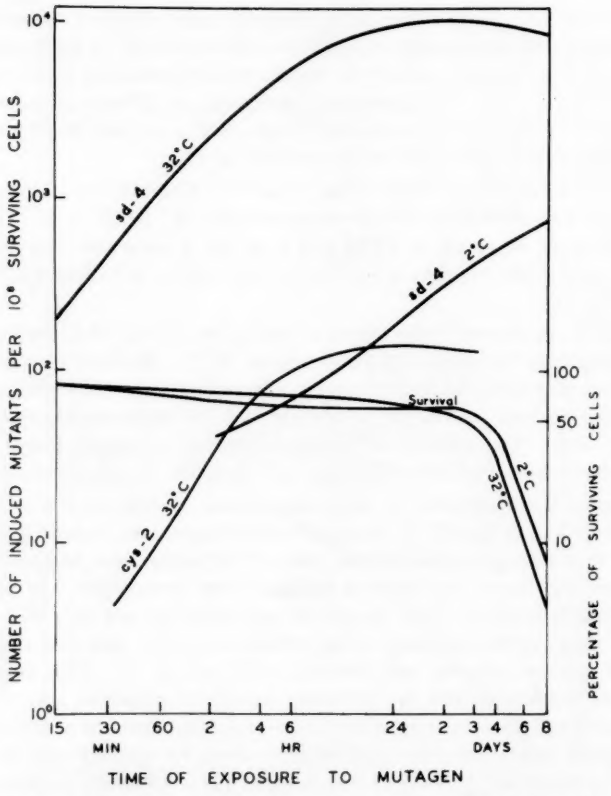

FIG. 3.—Effect of the time of exposure to azaserine (5 μg/ml) on the number of induced mutants (*sd-4* and *cys-2* reversions) and on the percentage of survivors at 32° and 2°.

Further experiments were aimed at determination of the fate of the mutagen and the stability of the new mutation before its expression.

Effect of Retreatment with Freshly Prepared Mutagen (*Fig. 4*).—The purpose of these experiments was to examine the possibility that mutagen might either be used up or decay during the treatment and that its renewal might increase the mutagenic response.

The production of mutants was first followed for 3–4 hours at 32° C., after which period the suspension was divided in half. The cells in one portion were centrifuged and washed and immediately resuspended in the same volume of freshly prepared mutagen solution at 32° C., after which periodic assays on both suspensions were resumed. As may be seen in Figure 4, this procedure had no effect on the action of TEM, while it increased the mutagenicity of azaserine by a factor of 3–4, a result which points to the instability of azaserine in contact with the bacterial suspension under these conditions.

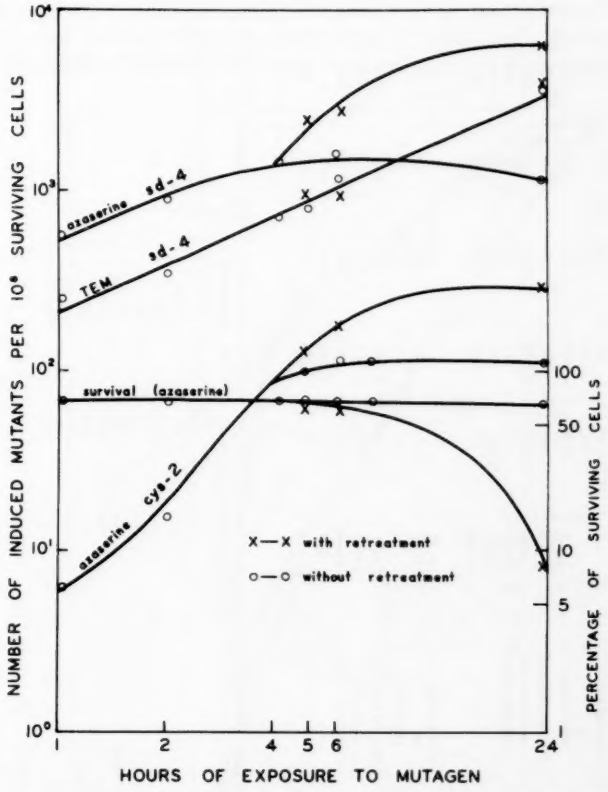

FIG. 4.—Effect of retreatment with freshly prepared mutagen (TEM 10 μg/ml; azaserine 3 μg/ml) on the number of induced mutants (*sd-4* and *cys-2* reversions); 32° C.

Effect of Various Posttreatments (*Tables 2 and 3*).—Following the demonstration that TEM- and azaserine-induced mutations could be conveniently studied in *E. coli*, attempts were made to analyze individual steps in mutagenesis with the aid of procedures capable of retarding, blocking, or reversing this process. The mutagen was removed from the cell environment and replaced with water or different salt solutions.

TABLE 2

EFFECT OF REMOVAL OF MUTAGEN AND POSTTREATMENT WITH DISTILLED WATER ON NUMBER OF MUTANTS INDUCED BY TEM (10 μG/ML), AZASERINE (5 μG/ML), AND $MnCl_2$ (400 μG/ML), AT 32° C.

MUTAGEN	TIME OF PREVIOUS EXPOSURE TO MUTAGEN		MUTANT	—INTERVAL (HOURS) OF POSTTREATMENT IN WATER AFTER PREVIOUS EXPOSURE TO MUTAGEN— 0	0.5	1	2	4	6	8	24 hrs
TEM		Percentage		100	77	76	74	74	...	...	60
Azaserine		of		62	63	42	19	18	...	...	13
$MnCl_2$	2	survivors		100	88	73	43	23	...	...	6.7
TEM	hours	No. of induced	*sd-4*	400	780	720	670	680	...	...	880
Azaserine		mutants per	*sd-4*	2,000	2,200	2,300	3,500	1,300	...	...	1,600
Azaserine		10^8 survivors	*cys-2*	5	17	11	58	52	...	...	57
$MnCl_2$			*sd-4*	500	550	500	940	670	...	...	300
$MnCl_2$			*cys-2*	83	55	70	110	250	...	...	70
TEM		Percentage		2.2	3.1	2.5	2.0	1.3	1.5	...	2.3
Azaserine		of		47	43	40	29	27	26	33	28
$MnCl_2$	18	survivors		32	9.7	9.3	9.3	4.6	4.6	4.4	1.65
TEM	hours	No. of induced	*sd-4*	5,500	3,600	3,400	4,300	6,100	5,700	...	3,700
Azaserine		mutants per	*sd-4*	1,600	2,600	2,500	3,500	2,900	1,700	1,500	1,900
Azaserine		10^8 survivors	*cys-2*	47	65	62	120	130	130	80	80
$MnCl_2$			*sd-2*	250	1,100	800	800	240	180	190	310
$MnCl_2$			*cys-2*	23	105	50	21	23	18	12	<15

TABLE 3

EFFECT OF REMOVAL OF MUTAGEN AND POSTTREATMENT WITH SALTS OF MONO- AND BI-VALENT METALS ON NUMBER OF MUTANTS (*sd-4*) INDUCED BY 2 HOURS OF PREVIOUS TREATMENT WITH TEM OR AZASERINE AT 32° C.

POSTTREATMENT MEDIUM		INTERVAL (HOURS) OF POSTTREATMENT AFTER EXPOSURE TO: TEM (10 μG/ML) 0	1	4	24 hrs	AZASERINE (5 μG/ML) 0	1	4	24 hrs
Distilled water		70	62	52	19	74	44	41	26
0.1 *M* NaCl		...	66	89	29	...	54	33	8
0.1 *M* KCl	Percentage	...	62	56	30	...	50	44	12
0.1 *M* $MgCl_2$	of	...	55	57	26	...	16	7	5
0.1 *M* $CaCl_2$	survivors	...	71	54	20	...	50	1.3	1.1
0.1 *M* $ZnCl_2$		...	17	1.6	<0.01	...	<0.01	<0.01	<0.01
0.1 *M* $MnCl_2$		...	53	46	19	...	1.2	0.6	0.1
Distilled water		1,100	1,500	1,300	3,100	2,400	3,800	6,900	2,600
0.1 *M* NaCl	No. of	...	1,500	850	550	...	4,000	6,300	2,600
0.1 *M* KCl	induced	...	1,600	850	840	...	4,400	1,700	320
0.1 *M* $MgCl_2$	mutants	...	1,700	1,100	900	...	9,300	14,000	2,500
0.1 *M* $CaCl_2$	per	...	1,600	4,800	1,200	...	4,200	950	
0.1 *M* $ZnCl_2$	10^8 survivors	...	800	5,100	0*	...	0*	0*	0*
0.1 *M* $MnCl_2$		...	1,650	1,200	1,200	...	11,500	11,000	15,000

* Excessive killing prevented the determination of mutagenicity.

In the experiments summarized in Tables 2 and 3, cells were treated at 32° C. for either 2 hours or 18 hours. After treatment, they were centrifuged, washed free of extraneous mutagen, and suspended in distilled water or various salt solutions; assays were made periodically over a period of 24 hours. As can be seen from Table 2, the number of mutants could not be reduced by this procedure, designed to allow any unbound intracellular mutagen to leach out of the cell. Indeed, the number of mutants seemed to increase approximately twofold during the first few hours of this presumed leaching process. The 24-hour readings, however, on the average were not significantly changed in most experiments.

The experiments in which salt solutions provided the posttreatment followed a generally similar course during the first hours of "leaching." Some salts even slightly or moderately increased the mutagenic action of TEM or azaserine during

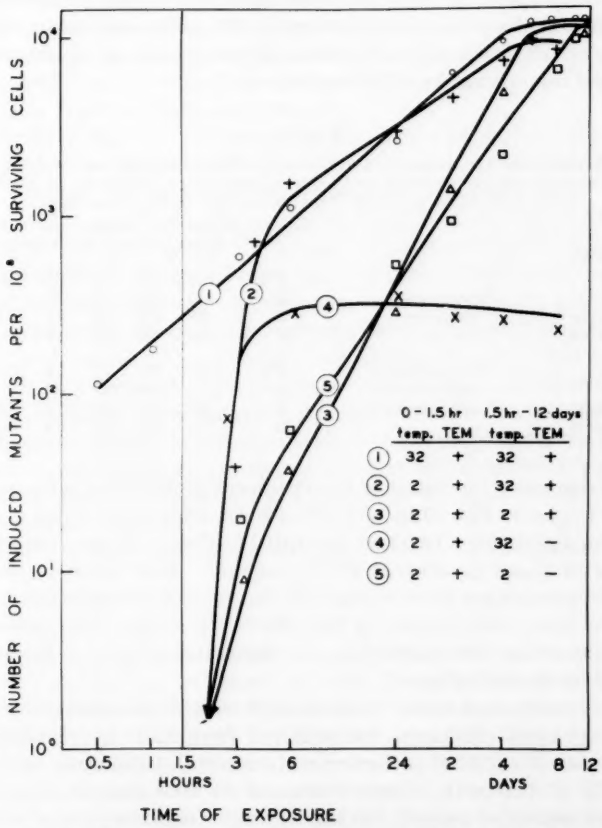

FIG. 5.—The effects of withdrawal of mutagen (TEM; 10 μg/ml) and change in temperature (2°, 32° C.) on the number of induced mutants (*sd-4* reversions). TEM was present throughout experiments 1–3 but only in the first hour and a half in experiments 4 and 5. Points represent one experiment, but comparable results were obtained in two other identical experiments.

1–4 hours of posttreatment. After 24 hours, however, the mutagenic effect in most cases was less than that obtained with distilled-water treatment. This was especially true for TEM, in which case most of the salts reduced the final number of mutants; only 0.1 KCl showed a considerable antagonistic effect against azaserine.

The most pronounced and permanent synergistic effect on the mutagenic action of azaserine was produced by $MnCl_2$, which, however, did not influence the mutagenicity of TEM. Magnesium ions caused a similar, although less permanent, effect. More detailed analysis of these interactions has been described elsewhere.[9]

Effect of Early Withdrawal of Mutagen (*Fig. 5*).—The results of earlier experiments had indicated that an appreciable increase in mutants could be expected when mutagen-treated cells were washed and held in water or salt solution. This increase was noted both at 32° and at 2° C. (Table 4). Experiments were therefore designed to study the effects of short-term exposure to mutagen at low temperature, followed by prolonged treatment in the same suspending medium (distilled water). TEM was selected as the mutagen because of the instability of azaserine evident from Figure 4 and Tables 2–4.

TABLE 4

EFFECT OF WITHDRAWAL OF MUTAGEN AND CHANGE IN TEMPERATURE ON NUMBER OF MUTANTS INDUCED (*sd-4* TO STREPTOMYCIN INDEPENDENCE) IN 18 HOURS, AT 2° C. BY EXPOSURE TO TEM (10μG/ML) OR AZASERINE (5 μG/ML)

MUTAGEN		INTERVAL (HOURS) AND TEMPERATURE OF POSTTREATMENT IN WATER AFTER PREVIOUS EXPOSURE TO MUTAGEN Temp.	0	0.5	1	2	4	6	24 hrs.
TEM	Percentage of survivors	2°C.	100	84	90	86	80	65	67
TEM		32	100	98	86	82	80	67	55
Azaserine		2	90	63	64	71	63	62	60
Azaserine		32	90	68	73	86	70	67	59
TEM	No. of induced mutants per 10^8 survivors	2°C.	80	80	72	75	105	110	170
TEM		32	...	139	194	184	166	177	264
Azaserine		2	11	24	27	28	38	45	44
Azaserine		32	...	36	59	48	40	61	35

Figure 5 represents the results of an experiment in which the cell suspension was treated for $1^1/_2$ hours with 10 μg. of TEM at 2° C., after which period it was divided into four equal portions. Two were incubated further in the presence of TEM, one at 2° C. (curve *3*) and the other at 32° C. (curve *2*). Both curves reached plateaus at about 10^4 mutants per 10^8 surviving cells, but curve *2* climbed much faster within the first few hours after transfer of the cells to 32° C. and then followed control curve *1* representing cells treated from the beginning at 32° C. These results are similar to those shown in Figure 2.

Somewhat paradoxical results were obtained with the remaining two portions of the cell suspension. Mutagen was removed from both by centrifugation and washing of the cells at 2° C., before reincubation in distilled water at 2° C. (curve *5*) and at 32° C. (curve *4*). Curve *5* followed the same general course as curve *3*. Curve *4* first resembled curve *2*, but the number of mutants soon became constant at a level approximately 30–40 times lower than that recorded for any of the other three portions. Thus cell suspensions exposed to mutagen at low temperature for only a limited period seemed to yield a higher number of mutants at low temperature than at 32° C.

COMMENTS

The experimental data here presented substantiate the previously suspected complexity of induced mutational processes. Diffusion of the mutagen into the cell is presumably the first step of the mutational event. For TEM and azaserine, diffusion proceeds unaided, while a violent osmotic decompression appears to be required to introduce $MnCl_2$ into the cell[2, 10] (Table 1). The observed maximum expression of mutagenicity after a rather short exposure to TEM at 2° C. (Fig. 5, curve *5*) is consistent with a high efficiency of diffusion at low temperatures. The same experiment also indicates the existence of a second step, a semireversible binding of the mutagen in a form in which it is not lost during subsequent incubation in distilled water at 2° C. and its effect is gradually expressed to the same degree as if the extraneous mutagen were not removed. Curve *4*, in the same experiment, however, suggests a partial reversibility of the second step at a higher temperature, as an explanation of the paradoxically lower final number of mutants at 32° C. These experiments, however, do not permit a definite distinction between true reversal with loss of intracellular mutagen and a secondary reaction that may involve the decay of some mutagenic principle.

The processes of diffusion and semireversible binding take place very rapidly at 2° and 32° C., but the next step, which is manifested as an increase in mutants after removal of the mutagen, is temperature-dependent to a high degree. Measurement of the rate of the third step is possible because of the nature of the assay system, in which the final number of mutant colonies is determined at the time the treated cells are plated on the selective agar medium. This step seems to be arrested when the cells are transferred from water to the solid growth medium lacking only one growth factor, where, as shown by earlier experiments,[2] they can undergo a few terminal divisions. The situation thus roughly resembles that encountered by Witkin[11] in her studies of ultraviolet-induced mutations, a short-term exposure to TEM at 2° C. being analogous to a mutagenic dose of irradiation; in both cases the final number of mutants is highly dependent on the events intervening between the exposure to mutagen and the end of the first cell division on the selective medium. Determination of the fate of intracellular mutagen awaits biochemical and radioactive tracer[10] studies.

We would like to acknowledge the very skilful technical assistance of Miss Clara E. Biro.

SUMMARY

Triethylene melamine and azaserine are mutagens particularly suited to the quantitative evaluation of mutagenic processes, because within wide limits of effective concentration the survival of treated cells is close to 100 per cent. The maximum yield of over 10^4 mutants per 10^8 surviving cells produced by TEM does not depend on temperature, although the rate of appearance of the mutants is much lower at 2° than at 32° C. Only a short period of exposure to TEM at low temperature is required to obtain subsequent full expression of mutagenicity in the absence of the mutagen at 2° C.; transfer to 32° C. causes the effect of the mutagen to be partially lost, presumably as a result of secondary decay of a mutagen-cell complex. The kinetic data point to the intricate nature of the mutagenic response with participation of the postulated steps: diffusion and semireversible binding,

which are rapid even at low temperatures, and a temperature-dependent reaction which proceeds at a comparatively low rate in a nutrient-free, aqueous medium. The events which follow transfer of the cells to solid media were not elucidated.

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[5] The term "back-mutant" requires some qualification. It was not attempted to determine whether the mutation to streptomycin-independence represents a true reversion or a suppressor-type mutation. The latter seems more likely in view of the slow initial growth and pronounced sectoring of the revertant colonies, and on the basis of recombination experiments with strain K-12 of *E. coli* (a cross between streptomycin-independent F^+ and F^- auxotrophs derived from streptomycin-dependent strains yielded a large proportion of streptomycin-dependent recombinants, indicating that, at least in this particular case, reversion to streptomycin-independence was a suppressor mutation; Szybalski, unpublished).

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TRIVIALITY OF VECTOR BUNDLES OVER THE AFFINE SPACE K^2

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In his paper "Faisceaux algébriques cohérents,"[1] J. P. Serre has proposed the problem: Is every algebraic vector bundle over the affine space K^n trivial? Or, equivalently, is every finitely generated projective module P over the ring of polynomials $K[x_1, \ldots, x_n]$ in n-variables free? For the case $n = 1$, it is well known that the answer is in the affirmative. In this note it is shown that the answer is still in the affirmative for the case $n = 2$; in fact, we prove that every finitely generated projective module over $A[x]$, the ring of polynomials in one variable over a principal ideal ring A, is free. The author is indebted to Professor J. P. Serre, who suggested the demonstration presented here after having seen the original "non-intrinsic" demonstration of the author for the case $A[x] = K[x_1, x_2]$, K algebraically closed.

Let σ be a commutative integrity domain with unit. We denote by $SL^*(n, \sigma)$ the group of inversible matrices of order n over σ with determinant ± 1 and by $SL(n, \sigma)$ its subgroup of matrices with determinant $+1$.

Proposition 1. Let A be a principal ideal ring and p a prime element of A (we often identify p and the ideal which it generates). Then the mapping

$$SL(n, A) \to SL\left(n, \frac{A}{p}\right)$$

is epijective (onto).

For $n = 1$, this is trivial. By the structure of matrices over a Euclidean ring,[2] it is easily seen that we have only to verify that diagonal matrices belonging to $SL(n, A/p)$ can be lifted. For $n = 2$, this is verified quite easily and is, in fact, classical when A is the ring of integers. The general case is easily reduced to the case $n = 2$.

Proposition 2. Let A be a principal ideal ring and p a prime element. Then the mapping

$$SL(n, A[x]), \to SL(n, A/p[x])$$

induced by the canonical projection $A[x] \to A/p[x]$ is epijective.

This is an immediate consequence of the previous proposition, since the diagonal elements of a diagonal matrix $M \in SL(n, A/p[x])$ belong to A/p.

Remark. Propositions 1 and 2 are immediate when A is the ring of polynomials in one variable over an algebraically closed field.

THEOREM. *Let A be a principal ideal ring and $A[x]$ the ring of polynomials in one variable over A. Let P be a finitely generated projective module over $A[x]$. Then P is free.*

Let K denote the field of quotients of A. The module $P \otimes_{A[x]} K[x]$ is a projective module over $K[x]$.[3] Since $K[x]$ is a principal ideal ring, $P \otimes K[x]$ is a free module over $K[x]$ with a finite number of generators. Let $\{x_i\}$, $i = 1, \ldots, n$, be a base. The x_i's have the form $x_i = y_i \otimes \frac{1}{a_i}$, $y_i \in P$, $a_i \in A$. The submodule L of P generated by y_i's is free. Let $\{o_i\}$ $(i = 1, \ldots, m)$ be a set of generators of P over $A[x]$. $o_i = (\sum_j \lambda_{ij} y_j) \otimes \frac{1}{b_i}$, where $\lambda_{ij} \in A[x]$ and $b_i \in A$. If $a \in A$ denotes the least common multiple of the b_i's, we have the relation

$$aP \subset L \subset P, \qquad a \in A.$$

We can assume that this decomposition is "maximal in the sense that (*i*) if a_1 is a *proper* divisor of a, $a_1 P$ is not contained in L; and (ii) if L_1 is a *free submodule* of P with the property $aP \subset L \subset L_1 \subset P$, then $L_1 = L$. The first assumption is trivial. The second one follows from the Noetherian character of the module P (every increasing sequence of submodules of P is stationary).

If a is a unit of A, $L = P$ and there is nothing to prove. If a is not a unit, a has a prime divisor p ($p \neq 1$ by the definition of a prime element). Consider the quotient modules L/pL and P/pP.

$$L/pL = L \otimes_{A[x]} A/p[x], \qquad P/pP = P \otimes_{A[x]} A/p[x].$$

L/pL and P/pP are projective modules over $A/p[x]$.[4] Since $A/p[x]$ is a principal ideal ring, these modules are free. There is a natural mapping i^*

$$i^*: L/pL \to P/pP.$$

We note that the kernel of the mapping $L \rightarrow P/pP$ obtained by composing the canonical projection $L \rightarrow L/pL$ and i^* is $L \cap pP$. Let N_p and I_p denote the kernel and image of i^*, respectively. We have the following exact sequence:

$$0 \rightarrow N_p \xrightarrow{j} L/pL \xrightarrow{i^*} I_p \rightarrow 0.$$

N_p and I_p are again free modules over $A/_p[x]$. Therefore, the exact sequence splits.

We say that $N_p \neq 0$; for let $a = pa'$, then $a'P$ is not contained in L. Therefore, there exists $x \in P$ such that $a'x \notin L$. Let $z = ax$. Then $z = pa'x$ so that $z \in pP \cap L$; but $a'x \notin L$; hence $z \notin pL$. Thus $pP \cap L \neq pL$, so that the kernel N_p of $L/pL \rightarrow P/pP$ is not zero.

Since the above exact sequence splits, it is possible to choose a basis $\bar{e}_1, \ldots, \bar{e}_k, \bar{e}_{k+1}, \ldots, \bar{e}_n$ of L/pL over $A/p[x]$ such that $j^{-1}(\bar{e}_1), \ldots, j^{-1}(\bar{e}_k)$ constitute a basis of N_p and $i^*(\bar{e}_{k+1}), \ldots, i^*(\bar{e}_n)$ constitute a basis of I_p over $A/p[x]$ $(k \geq 1)$.

If $\{\varepsilon_i\}$ is a basis of L over $A[x]$, then $\{\bar{\varepsilon}_i\}$, $\bar{\varepsilon}_i$ being the image of ε_i under the canonical projection $L \rightarrow L/pL$, form a basis of L/pL over $A/p[x]$. Therefore, there exists an invertible matrix M_1 over $A/p[x]$ such that $M_1 \cdot \bar{\varepsilon}_i = \bar{e}_i$. The determinant of M_1 is a non-zero constant belonging to A/p. Let $\bar{M}$ be the matrix which is the same as M_1 but for the first row, which differs from that of M_1 by a multiplicative constant belonging to A/p, so that $\det \bar{M} = +1$. Define $\{\bar{e}_i'\}$ by the relation $\bar{M} \cdot \bar{\varepsilon}_i = \bar{e}_i'$. The system $\{\bar{e}_i'\}$ has the same property as $\{\bar{e}_i\}$. We can therefore assume that $\bar{M}$ is a matrix belonging to $SL(n, A/p[x])$ with the property $\bar{M} \cdot \bar{\varepsilon}_i = \bar{e}_i$. By Proposition 2, there exists a matrix $M \in SL(n, A[x])$ which lifts $\bar{M}$. We define the set of elements $\{e_i\}$, $e_i \in L$ by the relation $M \cdot \varepsilon_i = e_i$. The system $\{e_i\}$ forms a base for L over $A[x]$; further, there exist $f_i \in P$ such that $pf_i = e_i$, $i \leq k$, since $\{e_i\}$, $i \leq k$, is in the kernel of $L \rightarrow P/pP$. Obviously, the elements f_i do not belong to L. The elements $f_1, \ldots, f_k, e_{k+1}, \ldots, e_n$ form a free submodule L_1 of P which contains L *strictly*. This contradicts the maximality of L. Therefore, the hypothesis that a is not a unit leads to a contradiction, which implies a is a unit and P is free.

COROLLARY. Let L be a free module with n generators over $A[x]$, A being a principal ideal ring. Let $\alpha = (a_1, \ldots, a_n)$ be an element of L such that there exist $p_1, \ldots, p_n \in A[x]$ with the property $\sum_i a_i p_i = 1$. Then there exists an invertible matrix M over $A[x]$ whose first row is $(a_1, \ldots, a_n)$.

Remark. It can be proved in the case of a polynomial ring in several variables that the corollary implies the theorem.

[1] J. P. Serre, "Faisceaux algébriques cohérents," *Ann. Math.*, **61**, 243, 1955.

[2] B. L. Van der Waerden, *Modern Algebra* (New York: Frederick Ungar Publishing Co., 1950), II, 106–108.

[3] H. Cartan and S. Eilenberg, *Homological Algebra* (Princeton, N. J.: Princeton University Press, 1956), chap. II, Proposition 6.1.

[4] *Ibid.*

THE RAPID COMPUTING MACHINE AS AN INSTRUMENT IN THE DISCOVERY OF NEW RELATIONS IN THE THEORY OF NUMBERS

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Any investigation in the theory of numbers is likely to be experimental, at least in its initial stages. The number theorist may study special cases of results which he may conjecture to be true. He may then find, of course, that his conjecture is not entirely correct. On the other hand, he may discover, as far as he is able to carry his computations, that the conjecture holds up, so he naturally likes to be able to use a rapid digital computing machine, or other means, to extend his computations. However, before the invention of any such machines, Euler, Gauss, Jacobi, Cauchy, and others of their time obtained some of the most important results we have concerning whole numbers. These men were expert computers and published papers containing extensive numerical data they had used in testing conjectures, which they were later able to prove or prove with modifications.

In much of the computing work being done at present, the man in charge has a definite and special problem in mind in some mathematical subject, pure or applied. He may wish to solve a differential equation, for example, or he may wish, having been given certain conditions, to determine some number, at least approximately. Others may wish to test some criteria given in connection with a new result. Often, in this sort of work, little except the final results are of value, and the incidental data are not tabulated. *Here we wish to discuss the application of tables where we have no special problem in mind but hope, by study of the tables derived, to be able to formulate a problem and be guided to some extent toward its solution.*

Examination of numerical tables tells the number theorist so often what *isn't* true. For example, before we were able to use extensive pertinent data recorded by computing machines, we had made conjectures as to the answers to a particular problem; but when more extensive computations were made, it turned out that a number of the conjectures were inaccurate. So a lot of time had been lost in trying to prove or disprove the false conjectures.

In addition, we hope, by study of the tables, to observe patterns indicating the existence of certain theorems which may turn out to be of an entirely novel character. For example, it is fairly clear that Euler must have discovered the Law of Quadratic Reciprocity by a study of the results of his extensive computations. There seems to be nothing in the literature which could have suggested it to him. He did not prove the law. This was achieved by Gauss, and this was the beginning of the development of a far-reaching subject in number theory.

The use of tables is also very valuable, of course, when we need checks on formulas, which we have apparently proved, involving elements in the tables.

The nature of some of our procedures with tables is best seen, we think, if we describe an investigation which was supported by the use of extensive tables either obtained by the use of tables of indices, or by the use of the SWAC, which Numeri-

cal Analysis Research at U.C.L.A. has kindly permitted use of at various times since 1953. *Of particular significance in connection with this is the fact that the research started on what appeared to be an entirely new problem.*[1] *As a consequence of this, practically everything we have discovered has seemed to be new.*

Let p be an odd prime integer. Then we discuss the following conditional congruence:

$$ax^m + by^k + d \equiv 0 \pmod{p}, \tag{1}$$

where a, b, d, x, $y \not\equiv 0 \pmod{p}$ with a, b, and d given integers and with x and y integers to be determined. Also, we shall assume that m and k are divisors, each > 1 of $p - 1$, since the problem for m and k arbitrary integers reduces to the problem involved when m and k each divide $p - 1$. In examining this congruence (1) for possible solutions x and y, each integers, it is sufficient to consider those values x_1, y_1 such that $0 < x_1 < p$ and $0 < y_1 < p$. Hence there is only a finite number of integers x and y that we need to examine in order to determine by trial all the possible solutions of congruence (1).

Since we have assumed $d \not\equiv 0 \pmod{p}$, we may multiply congruence (1) by d_1 where $dd_1 \equiv 1 \pmod{p}$, which is always possible, and obtain

$$a_1x^m + b_1y^k + 1 \equiv 0 \pmod{p}.$$

Hence it is sufficient to consider congruence (1) in the form

$$ax^m + by^k + 1 \equiv 0 \pmod{p}. \tag{1a}$$

In the examination of congruence (1a) it has so far been found convenient for many purposes to employ the fact that an integer, g, may be found corresponding to any odd prime p such that $g^{p-1} \equiv 1 \pmod{p}$, but $g^k \not\equiv 1 \pmod{p}$ for $0 < k < p - 1$. This being the case, we may reduce the problem stated in connection with congruence (1a) to the problem of finding the values of r and s in the relation

$$1 + g^{i+kr} \equiv g^{j+ms} \pmod{p}, \tag{2}$$

where $g^i \equiv b$, $g^j \equiv -a$, $g^r \equiv y$, $g^s \equiv x$, modulo p. By the properties mentioned for g we can take r in the set $0, 1, \ldots, (p - 1)/k$, and s in the set $0, 1, \ldots, (p - 1)/m$. We now represent by $[i, j]_{k,m}$ the number of sets of solutions (r, s) of congruence (2). To illustrate this, we may set up a table giving the values of each $[i, j]$ when $p = 31$, $k = 3$, $m = 3$, in congruence (2) as follows:

		j		
		0	1	2
	0	3	4	2
i	1	4	2	4
	2	2	4	4

Here $[i, j]_{k,m}$ is the element in the ith row and jth column; for example, $[2, 1]_{3,3} = 4$.

Also, if $p = 37$, $k = 3$, $m = 3$, we have

		j		
		0	1	2
	0	2	4	5
i	1	4	5	3
	2	5	3	4

We note for the top table above that each row adds to 10, except the first row, which adds to 9, and a similar situation holds for the columns. In the case of the second table, we find that each row adds to 12, except the first row, which comes to 11. *These illustrate a known general result which goes back, in special cases, to the time of Gauss, namely, that*

$$\sum_{j=0}^{m-1} [i, j]_{m,m} = \begin{cases} (p-1)/m \text{ if } i \not\equiv 0 \pmod{m}, \\ (p-1)/m) - 1 \text{ if } i \equiv 0 \pmod{m}. \end{cases} \tag{3}$$

Now, however, if we examine the tables as to divisibility of the elements, *we note that each element in the top table is even except* $[0, 0]_{3,3}$, *but this situation does not hold for the elements of the second table.* Then, if we set up a table corresponding to $p = 43$, $k = 3$, $m = 3$, we find again, as for the elements for the top table, that each element is even, except $[0, 0]_{3,3}$ corresponding to the results for the top table. If we take the value for m and k, 3 in each case, we find that this situation holds for $p = 43$, 127, and 157. However, if we take $p = 73$, $m = 3$, $k = 3$ we find that $[0, 0]_{3,3}$ is 8, *but each other element in the table corresponding is divisible by 3.*

The above results concerning the divisibility of certain $[i,j]$'s seem to point to the existence of a general theorem, which we have so far been able to prove only for certain special cases. Apparently, the use of some of the deepest results in the theory of cyclotomic fields is necessary in order to throw light on this problem. *This illustrates the extremes we may find in a study of such tables. In one case we are led to the relation* (3), *which is very easy to prove; and, on the other hand, an inspection of the tables as to the divisibility of their elements leads to difficult and quite new problems and results, and apparently there was nothing in the literature that could have suggested them.*

Suppose in congruence (2) we set $k = c$ with $mc = p - 1$. We now propose a problem which is quite general, as follows:

Problem: If in (1a) *a and b are fixed integers, m is fixed, and p increases together with c subject to the relation* $mc = (p - 1)$, *what can be said about the number of the sets of solution x, y?*

This problem was formulated in 1953 and is the one described previously as having been examined on the SWAC and by other means continuously since that time.[1] In 1954 Emma Lehmer coded the general problem of calculating the values of the $[i,j]$'s on the SWAC and checked all our previous tables (computed by the use of tables of indices) giving values of the $[i, j]$'s. In the spring of 1955 Nicol and Selfridge extended this program on the SWAC and tabulated all the values $[i, j]$ for $l = 5$, for each $p \equiv 1 \pmod{5}$ for $p < 1024$; also for $l = 7$ with all p's < 1024; $p \equiv 1 \pmod{7}$.

Now let us take in congruence (2), $p = 31$, $c = 6$, $m = 5$. Then our table of the $[i, j]_{6,5}$ is as follows:

		j				
		0	1	2	3	4
	0	1	2	1	1	0
	1	1	0	0	2	2
i	2	1	0	3	0	1
	3	1	0	0	2	1
	4	1	1	1	1	1
	5	0	3	1	0	1

We also have occasion to use the known general results,

$$\sum_{j=0}^{m-1} [i, j]_{c,m} = m - \begin{cases} 0 \text{ if } i \not\equiv c/2 \pmod{c}, \\ 1 \text{ if } i \equiv c/2 \pmod{c}, \end{cases} \tag{4}$$

and, if m is even,

$$\sum_{j=0}^{m-1} [i, j]_{c,m} = m - \begin{cases} 0 \text{ if } i \not\equiv 0 \pmod{c}, \\ 1 \text{ if } i \equiv 0 \pmod{c}. \end{cases} \tag{5}$$

In view of the last two relations, we note the two extreme cases: first, when all the $[i, j]$'s are zero except one and that equals m, which we shall call[2] Case A; second, the case where each $[i, j]$ is unity, which we shall call Case B. Special attention is given to these cases because of a connection with Fermat's Last Theorem.

Examination of our tables for the case when $m = 3$ led us to suspect the following results: If Case A holds for a given i in congruence (2), then Case B holds for $(-i)$ in lieu of i in the two cases, first when c is divisible for m, and second when c is prime to m. It turned out that this result is very easy to prove; in fact, the idea is so obvious that perhaps that is the reason it was overlooked in the period before we studied the tables.

We now examine other results to which we were led, mainly by an examination of the extensive tables. It turned out that, for a given $p = 1 + mc$ in congruence (2), approximately one-third of the elements appearing in our tables were zero.[3] For about 70 per cent of these cases where $[i, j]$ was zero we were enabled to prove that an infinity of Diophantine equations in x and y with integral coefficients had no solutions. Following this line of thought, we were also able to prove the following:

THEOREM. *The equation*[4]

$$da + k_1 p + (db + k_2 p)x^c = (d + k_3 p)y^l,$$

with $(a, b) = 1$; *a, b, and l fixed, is impossible for an infinity of integers c such that* $cl + 1 = p$ *with p and l odd primes,*

$$\frac{a^l + b^l}{a + b} \neq r^l; \qquad a + b \neq s^l; \qquad a \neq t^l,$$

where a, b, r, s, t are integers, $d \not\equiv 0 \pmod{p}$; *also where* k_1, k_2, *and* k_3 *are integers.*

If we consider again our tables giving values of each $[i, j]$ in congruence (2) with

$k = c$, $m = l$, and $mc = p - 1$, an inspection of them makes it appear likely that if we consider a partition of m as the sum of integers in the set $0, 1, \ldots, m - 1$, then, for c sufficiently large, at least one such partition appears, as a row in the corresponding table for this c and m. *This led to a proof of a special case of this conjecture which appears deeper than any of the results mentioned above.*

In appraising the value of the tables above, it is well to examine, of course, the connections that the known theory behind them has with other subjects. In this case the theory of the $[i, j]$ symbols is included in the theory of the conditional equation in a finite field, that is, the equation

$$f(x_1, x_2, \ldots, x_n) = 0,$$

where f is a polynomial with coefficients in a finite field $F[p^n]$, with p prime, and our problem is to determine properties of the x_i. The importance of this theory and its connection with other subjects is now well known.

Finally, we shall illustrate a statement made near the beginning of this article to the effect that often in working with a special problem on a rapid computing machine the investigator is interested in little except the final result, and the incidental data obtained in the course of computation are not tabulated. The statement of Fermat (known as Fermat's Last Theorem), which dates from 1672, that

$$x^n + y^n = z^n, \qquad n > 2,$$

has no solutions in integers, each non-zero, still awaits a proof or disproof. It is known that for a proof it is sufficient to consider the case of $n = l$, an odd prime. In 1954 and 1955 D. H. Lehmer, Emma Lehmer, C. A. Nicol, J. L. Selfridge, and H. S. Vandiver tested criteria for the solution of the above equation on the SWAC and proved that the said equation did not hold in non-zero integers for any prime exponent $n > 2$ and < 4002. In the several articles we published where this result was discussed, limited tables were included from the computations on the SWAC, which gave us, however, much information concerning particular cyclotomic algebraic fields, as brought out by the writer in another paper. On the other hand, in addition to the above, we might have tabulated much more information from the data obtained having to do with the divisibility of the numerators of Bernoulli numbers (in their lowest terms). However, we did not deem this fact of sufficient importance to merit the making of typewritten or printed tables. Contrary to this, the tables we discussed in the first part of our paper we deem to be of a type which merits publication of them.

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[1] The problem was originally stated by Erna H. Pearson and H. S. Vandiver, "On a New Problem Concerning Trinomial Congruences Involving Rational Integers," these PROCEEDINGS, **39,** 1278–1285, 1953. Some results obtained from researches on this problem were given in the following articles by the present author, all of which appeared in these PROCEEDINGS; "New Types of Trinomial Congruence Criteria Applying to Fermat's Last Theorem," **40,** 248–252, 1954; "On Trinomial Equations in a Finite Field," **40,** 1008–1010, 1954; "On the Properties of Certain Trinomial Equations in a Finite Field, **41,** 651–653, 1955; "Relation of the Theory of Certain Trinomial Equations in a Finite Field to Fermat's Last Theorem," **41,** 770–775, 1955; "Diophantine Equations in Certain Rings," **42,** 656–665, 1956. See also C. A. Nicol, J. L. Selfridge, and H. S. Vandiver, "On Diophantine Equations Which Have No Solutions," these PROCEEDINGS, **42,** 264–266,

1956; Emma Lehmer and H. S. Vandiver, "On the Computation of the Number of Solutions of Certain Trinomial Congruences," *J. Assoc. Computing Machinery*, **4**, 505–510, 1957.

[2] For $p < 1024$ and $l = 5$, Case A occurred only for $p = 521$, 881, 941, and 971. Also for $p < 1024$ and $l = 7$, Case A did not appear at all. This situation suggests the possibility of the least value of p for which Case A holds may, in general, increase with l; so it might happen that, for l comparatively small, it would tax the resources of our largest digital computing machines, if we attempted to find the smallest corresponding p. However, it is possible to show that such a p always exists for a given l; in fact, there is an infinity of p's satisfying the conditions for Case A. If such a situation holds, it gives quite a sidelight on certain types of mathematical induction.

[3] We may throw some light on the possible number of zero values using the relation if i ranges over $0, 1, \ldots, c-1$ and j over $0, 1, \ldots, l-1$; $d = (c, l)$

$$\sum_{i,j} [i, j]^2 = 2cl - c - l + (1 - d)$$

(these PROCEEDINGS, **35**, 683–684, 1949; there is a misprint on p. 683 in the first equation given at the bottom of the page. The left-hand member should read $\sum_{i,j} m_1 m_2 [i, j]^2$, instead of as printed). The other quadratic relations given on p. 683 might also be used in this connection.

[4] This result illustrates an approach to Diophantine equations which might be new, but similar points of view in other parts of mathematics are common. Usually in Diophantine analysis the approach, in case we consider one equation in two unknowns, is to write

$$f(x, y) = c,$$

where the left-hand member is a polynomial with *fixed* rational integral coefficients and *fixed* integral exponents with x and y unknowns. In the present theorem we keep the integers a and b fixed, but k_1, k_2, k_3 are arbitrary integers with d any integer satisfying $d \not\equiv 0 \pmod{p}$. We also let the exponent c vary with l fixed, so that $cl + 1 = p$. This is an illustration of something that has been written before in connection with similar situations (Pearson and Vandiver, these PROCEEDINGS, **39**, 1278–1285, 1953): "If we have a conditional relation involving several given parameters it may be impossible to say much about the properties of the relation for the parameters all fixed; however, if we fix some and let some others range over a certain set of values we are often able to make some definite statements about the resulting set of relations."

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*ON THE INVARIANCE OF OPENNESS**

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1. *Introduction.* In a recent paper[1] attention was called to the desirability of subjecting to further study those spaces in which the property of being open is a topological invariant for subsets of the space. Euclidean spaces and manifolds have this property by the well-known theorem of Brouwer.[2] Thus it seems appropriate to say that a topological space X *has the Brouwer Property*, provided that any subset of X which is homeomorphic with an open subset of X is itself necessarily open. In this note an invariance theorem concerning the Brouwer Property under certain types of mappings will be established which yields not only the existence of spaces other than Euclidean manifolds having this property but also a method of generating such spaces in a considerable variety of ways.

The letters X and Y will denote spaces, and, in general, they will be assumed to *locally connected generalized continua*, that is, separable metric spaces which are

connected, locally connected, and locally compact. If U is an open set in such a space, its boundary $\bar{U} - U$ will be denoted by $Fr(U)$, and a connected open set will be called a *region*.

2. LEMMA. *If U is an open subset of a locally connected generalized continuum X and K is an open subset of $Fr(U)$ such that no subset of K separates any region in X, then $U + K$ is open in X.*

Proof. Let $p \in K$ and let R be a region in X about p small enough that $\bar{R} \cdot Fr(U) \subset K$, i.e., so that $\bar{R} \cdot [Fr(U) - K] = 0$. The set $Q = R - K \cdot R$ is then connected and intersects U because $p \in Fr(U) \cdot R$. On the other hand, $Q \cdot Fr(U) = 0$ by the choice of R. This gives $Q \subset U$, so that $Q + K \cdot R = R \subset U + K$; and, since R is open in X, p is interior to $U + K$. As p was an arbitrary point of K and U is open in X, $U + K$ is open in X, as asserted.

3. THEOREM. *Let $f(X) = Y$ be a mapping where X and Y are locally connected generalized continua. If there exists a closed set F in Y such that no locally compact set homeomorphic with a subset of F separates any region in Y and such that f is locally topological on $X - f^{-1}(F)$, then if X has the Brouwer Property, so also has Y.*

Proof. Let U be a subset of Y which is homeomorphic with an open set V in Y, and let $h(V) = U$ be a homeomorphism. Let p be a point of U which is on neither F nor $h(F)$. Then p must be an interior point of U. For let $z \in f^{-1}(p)$, $t \in f^{-1}(q)$, where $q = h^{-1}(p)$. Then, since f is locally topological at z, there exists an open set G in X about z such that $f|G$ is topological and $E = f(G)$ is open in Y. Likewise, since f is locally topological at t, there exists an open set W in X about t such that $f|W$ is topological, the set $Q = f(W)$ is open in Y, $Q \subset V$, and the set $R = h(Q)$ is contained in the open set E, so that we have

$$h(Q) = R \subset U \cdot f(G) = U \cdot E.$$

Now for each $x \in W$ there is a unique point $g(x)$ of $f^{-1}hf(x)$ in G; and the transformation g of W onto $S = f^{-1}(R) \cdot G$ thus defined is a homeomorphism, since $S \subset G$, $R \subset E$, and $Q \subset V$ and the mappings $f: G \to E$, $h: Q \to R$, and $f: W \to Q$ are topological. Accordingly, S is homeomorphic with W, and hence S must be open by the Brouwer Property of X. Thus R is open, because f is locally topological at all points of S and $R = f(S)$. Since $p \in R \subset U$, this shows that p is interior to U.

Next let p be a point of U which is on $h(F)$ but not on F. Since U is locally compact, there exists an open set W in Y about p with $W(\bar{U} - U) = 0$ and $\bar{W} \cdot F = 0$. Let $U' = U \cdot W$, and let U_0 be the set of all interior points of U'. As shown above, U_0 contains all points of U' except possibly points belonging to $h(F)$. Thus the set $K = U' - U_0$ is an open subset of $Fr(U_0)$, no subset of which can separate a region in Y. [*Note:* $Fr(U_0) \subset U' + (Y - W)$, since $Fr(U) \subset (Y - W) + U$.] Accordingly, by the lemma, $U_0 + K = U'$ is open in Y. Hence p is interior to U.

Now, if I denotes the set of all interior points of U, we have already shown that $U - I = K \subset F$. Thus $Fr(I) \subset K + (\bar{U} - U)$; and, since no point of U is a limit point of $\bar{U} - U$ by local compactness of U, this shows that K is an open subset of $Fr(I)$. Hence, by the lemma, $I + K = U$ is open in Y, as required by our theorem.

4. *Consequences. Examples.* A locally connected generalized continuum no region of which is separated by any set of dimension $\leq n - 2$ is said to be *locally a Cantorian manifold of dimension* $\geq n$. By an earlier theorem of the author[3] the

property of being such a manifold is invariant under light open mappings. In conjunction with this result, our theorem above yields at once:

COROLLARY 1. *Let X be locally a Cantorian manifold of dimension $\geq n$ and let $f(X) = Y$ be a light open mapping which is locally topological except at a subset F of X such that both F and $f(F)$ are of dimension $\leq n - 2$. Then if X has the Brouwer Property, so also has Y.*

Example 1. Let X be the Euclidean E_3, and let f be the natural mapping of the decomposition of E_3 into the single point (0, 0, 0) and the point pairs (x, y, z), $(-x, -y, -z)$. Then Y has the Brouwer Property but is not a Euclidean manifold.

COROLLARY 2. *Let f be closed and monotone and have only one non-degenerate point inverse K and suppose neither K nor any single point separates a region in X. Then Y has the Brouwer Property if X has it.*

Example 2. In $X = E_3$ let K be a circle. Let f be the natural mapping of the decomposition of E_3 into K and single points of $E_3 - K$. Then Y has the Brouwer Property. However, Y is not a Euclidean manifold, because every neighborhood of $y_0 = f(K)$ in Y contains 1-cycles in $Y - y_0$ (namely, f images of 1-circles linking K in E) which do not bound in $Y - y_0$.

Example. 3. Let X be the projective plane, and let K be a projective line in X. Define f and Y as in Example 2 (i.e., by decomposition). Then Y has the Brouwer Property, since K does not separate arbitrarily small regions about itself in X. Since Y is a sphere in this case, this is interesting but hardly surprising!

Example 4. A 2-dimensional space having the Brouwer Property may be constructed directly as follows. Join a torus T_1 to a second torus T_2 half its size along the points of a common circle and delete the interior points of this circle from each torus. Next join T_2 similarly to a torus T_3 half its size, so that the common circle to T_3 and T_2 is opposite (on T_2) to the circle common to T_2 and T_1. Join on T_4 to T_3 similarly and continue indefinitely in such a way that the sequence T_1, T_2, . . . converges to a point p not on $\sum T_n$. The set T obtained by adding p to the retained parts of T_1, T_2, . . . is not a 2-manifold, but it does have the Brouwer Property, as can be verified by an elementary, though non-trivial, argument.

Note. In the theorem it is not enough to assume that F itself and its subsets fail to separate any region in Y. For if we map a sphere (or S_n) onto a hemisphere by vertical projection, no subset of the created edge separates any region on the hemisphere, but the hemisphere does not have the Brouwer Property. Note here that *homeomorphs* of the edge *do separate* regions on the hemisphere.

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l-TYPE DOUBLING IN NH_3*

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I. *Introduction.*—Coriolis resonance interactions in polyatomic molecules have been studied extensively.[1-17] They arise from a term in the first-order Hamiltonian for the molecule $\sum_\alpha p_\alpha P_\alpha / I_{\alpha\alpha}$, where α is summed over x, y, and z, these being the body-fixed axes of the molecule, and where P_α is the αth component of the angular momentum of the molecule, $I_{\alpha\alpha}$ is one of the three principal moments of inertia, and p_α is the αth component of the "incipient" angular momentum,

$$\sum_{s\sigma < s'\sigma'} \zeta_{ss'}^{(\alpha)} \left[\left(\frac{\omega_{s'}}{\omega_s} \right)^{1/2} q_{s\sigma} p_{s'\sigma'} - \left(\frac{\omega_s}{\omega_{s'}} \right)^{1/2} q_{s'\sigma'} p_{s\sigma} \right],$$

due to two vibrations, ω_s and $\omega_{s'}$, bearing the appropriate symmetry relations to each other.[4] Here $\zeta_{ss'}^{(\alpha)}$ is known as the Coriolis coupling factor and depends upon the nature of the normal co-ordinates. When the frequencies ω_s and $\omega_{s'}$ are sufficiently close together, resonance sets in and the effects of such resonances were first observed in connection with the spectrum of the asymmetric molecule H_2CO,[5] but has since been observed in many other instances, including the spectra of axially symmetric molecules.[6-11]

The effects are essentially of two kinds in axially symmetric molecules, namely, where ω_t and $\omega_{t'}$ are two perpendicular frequencies and where one of the frequencies, say ω_t, is a perpendicular and where the other, say ω_s, is a parallel frequency. In the former case, $\zeta_{tt'}^{(z)} \neq 0$, while $\zeta_{tt'}^{(x)} = \zeta_{tt'}^{(y)} = 0$; in the other instance $\zeta_{ts}^{(z)} = 0$, while $|\zeta_{ts}^{(x)}| = |\zeta_{ts}^{(y)}| \neq 0$. In the former example, it would be the x component of ω_t and the y component of $\omega_{t'}$ that would resonate and vice versa. In the second case both the x and the y component of the frequency ω_t could resonate with the frequency ω_s, which would be along z. The first example has been studied in detail theoretically for axially symmetric molecules by Nielsen[12] and by de Heer[13] and by Lord,[8] who have applied it to certain bands in the spectrum of C_3H_4. The second example is considerably more complicated and has been discussed by Nielsen[14-17] in connection with the spectra of pyramidal molecules, particularly AsH_3, PH_3, and SbH_3. In this case, it is the parallel frequency ω_2 and the perpendicular frequency ω_4 which resonate. We shall here wish to consider it in relation to the band ω_4 in NH_3, especially the giant *l*-type splitting of the state $K = 1$ which is observed to exist.

II. *The Theoretical Consideration.*—According to Nielsen,[12] the term $(p_x P_x - p_y P_y)/I_{xx}$, $(I_{xx} = I_{yy})$, where p_x and p_y contain only the incipient part of the vibrational angular momentum identifiable with ω_2 and ω_4, has the following off-diagonal matrix element:

$$\begin{aligned}(v_s - 1, v_t, l_t, K | H^{(1)}/hc | v_s, v_t - 1, l_t \pm 1, K \pm 1) \\ = (v_s, v_t - 1, l_t \pm 1, K \pm 1 | H^{(1)}/hc | v_s - 1, v_t, l_t, K) \\ = \frac{1}{2} \left[\frac{\omega_t + \omega_s}{(\omega_s \omega_t)^{1/2}} \right] [v_s(v_t \mp l_t)]^{1/2} [(J \mp K)(J \pm K + 1)]^{1/2} \zeta_{s,t}^{(x)} B_e^{(xx)}, \quad (1)\end{aligned}$$

where v_t and v_s are the total vibration quantum numbers, l_t is the quantum number of angular momentum identifiable with the vibration ω_t, and J and K are, respectively, the quantum numbers of total angular momentum and of the component of the total angular momentum directed along the z-axis. Applied to the two perturbing states ω_2 and ω_4, where, respectively, $v_s = 1$, $v_t = l_t = 0$, and $v_s = 0$, $v_t = |l_t| = 1$, the energies are obtained from solving the secular determinant of the matrix which has not only the usual diagonal elements but also the off-diagonal elements (1). The secular determinant breaks up into submatrices, a submatrix for each value of J which has $3(2J + 1)$ rows and columns. The elements of such a submatrix may be arranged so as to further split up into other submatrices, of which the general form will be

	$v_t=1,\ l_t=1,\ v_s=0$ $K=K+1$	$v_t=l_t=0,\ v_s=1$ $K=K$	$v_t=1,\ l_t=-1,\ v_s=0$ $K=K-1$
$v_t=1,\ l_t=1,\ v_s=0$ $K=K+1$	$K+1+\epsilon$	$\left[\frac{(J-K)(J+K+1)}{2}\right]^{1/2}\alpha$	0
$v_t=l_t=0,\ v_s=1$ $K=K$	$\left[\frac{(J-K)(J+K+1)}{2}\right]^{1/2}\alpha$	$-\Delta+\epsilon$	$\left[\frac{(J+K)(J-K+1)}{2}\right]^{1/2}\alpha$
$v_t=1,\ l_t=-1,\ v_s=0$ $K=K-1$	0	$\left[\frac{(J+K)(J-K+1)}{2}\right]^{1/2}\alpha$	$-K+1+\epsilon$

(2)

where

$$\Delta = \{\delta - [B_e^{(zz)} - B_e^{(xx)}]\}/2\{[1 - \zeta_t^{(z)}]B_e^{(zz)} - B_e^{(xx)}\},$$

$$\alpha = \left[\frac{(\omega_s + \omega_t)}{(\omega_s\omega_t)^{1/2}}\right][\zeta_{s,t}^{(x)}][B_e^{(xx)}]/2\{[1 - \zeta_t^{(z)}]B_e^{(zz)} - B_e^{(xx)}\},$$

$$\epsilon = \frac{T(v) + J(J+1)B_e^{(xx)} + (K^2 - 1)[B_e^{(zz)} - B_e^{(xx)}] - \epsilon'}{2\{[1 - \zeta_t^{(z)}]B_e^{(zz)} - B_e^{(xx)}\}},$$

with $\delta = (\omega_t - \omega_s)$, $T(v)$ equal to the vibration term value and ϵ' equal to the perturbed energy (in the same units as $T[v]$). The value of $K - 1$ may not exceed J; neither may $K + 1$ be less than $-J$. Evidently, then, there will be two identical roots obtained by setting $K + 1 = -J$ and $K - 1 = J$ in equation (2); two identical subdeterminants of two rows and columns obtained by setting $K + 1 = -J + 1$ and $K - 1 = J - 1$ in equation (2) and $(2J - 1)$ other subdeterminants of three rows and columns each. $(J - 1)$ of the latter are repeated twice, and one (i.e., where $K + 1 = 1$) is unique. This is illustrated for the case $J = 2$ in Figure 1. The levels remain degenerate, to this approximation, in the quantum number K and l except for the state where $K + 1 = 1$. The subdeterminant referred to earlier where $K + 1 = 1$ is of particular interest because it removes the degeneracy of the levels $K = l = 1$ and $K = l = -1$. The splitting is entirely similar to the type of splitting usually referred to as l-type doubling. The essential difference is that here the perturbing frequencies are so close together that resonance exists, and for this reason it is a first-order effect, i.e., a giant l-type doubling.

The subdeterminant $K + 1 = 1$ can be factored into the three roots

$$\epsilon_0 = -1,$$

$$\epsilon_{\pm} = -\frac{(1 - \Delta)}{2} \pm \left[\left(\frac{1 + \Delta}{2}\right)^2 + \alpha^2 J(J+1)\right]^{1/2}, \qquad (3)$$

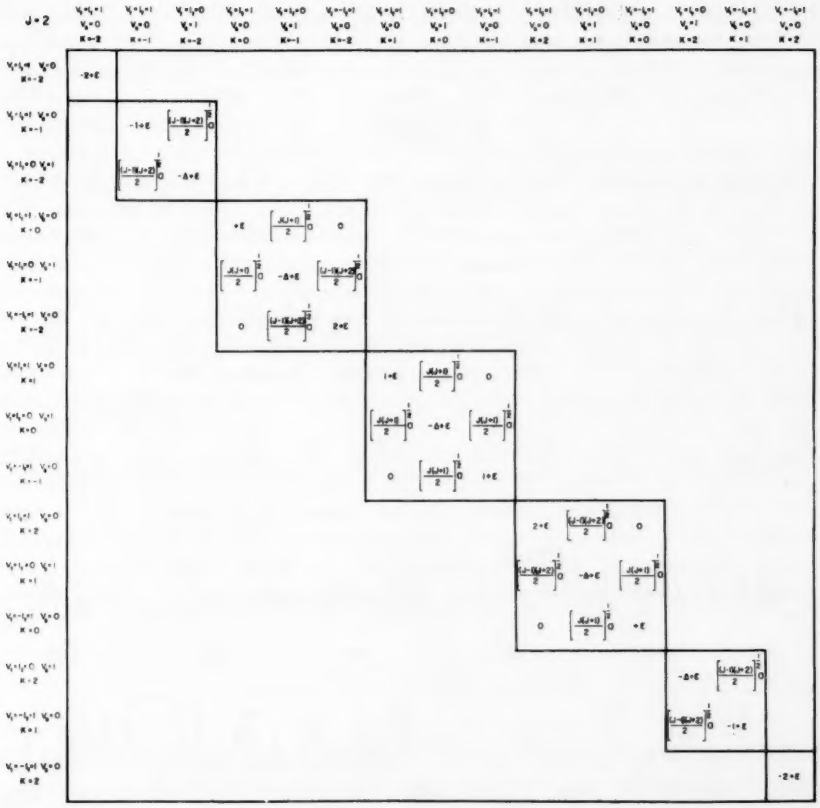

FIG. 1.—The submatrix for $J=2$

the values ϵ_0 and ϵ_- belonging to the perpendicular band, ω_4, and ϵ_+ belonging to the parallel band, ω_2. The selection rules are such that when the molecule makes a transition from the state $K = 0$ in the lower state to the state $K = 1$ in the upper state, the selection rules for J are: $\Delta J = 0$ is to the component ϵ_0 and $\Delta J = \pm 1$ is to the component ϵ_-. This will have the effect on the $K = 1$ subband of the perpendicular band ν_4 that the P and R lines will be shifted toward higher frequencies, while the Q branch will be shifted toward lower frequencies. For all the other values of K the effect is essentially that the ζ_{tl} value for a level becomes a function of the value of K of that level.

III. *Experimental Results.*—The effect of the perturbation discussed in Section II was observed in PH_3, AsH_3, and SbH_3 only to the extent that the dependence of $\zeta_{tl} = \zeta_4$ on K was observed. The l-type doubling was not observed in these molecules because of the greater overlapping of the K substructure which occurs in the fundamental bands. The measurements for the 6 μ band of ammonia were made using a vacuum prism-grating spectrometer discussed briefly by Nielsen[18] and in

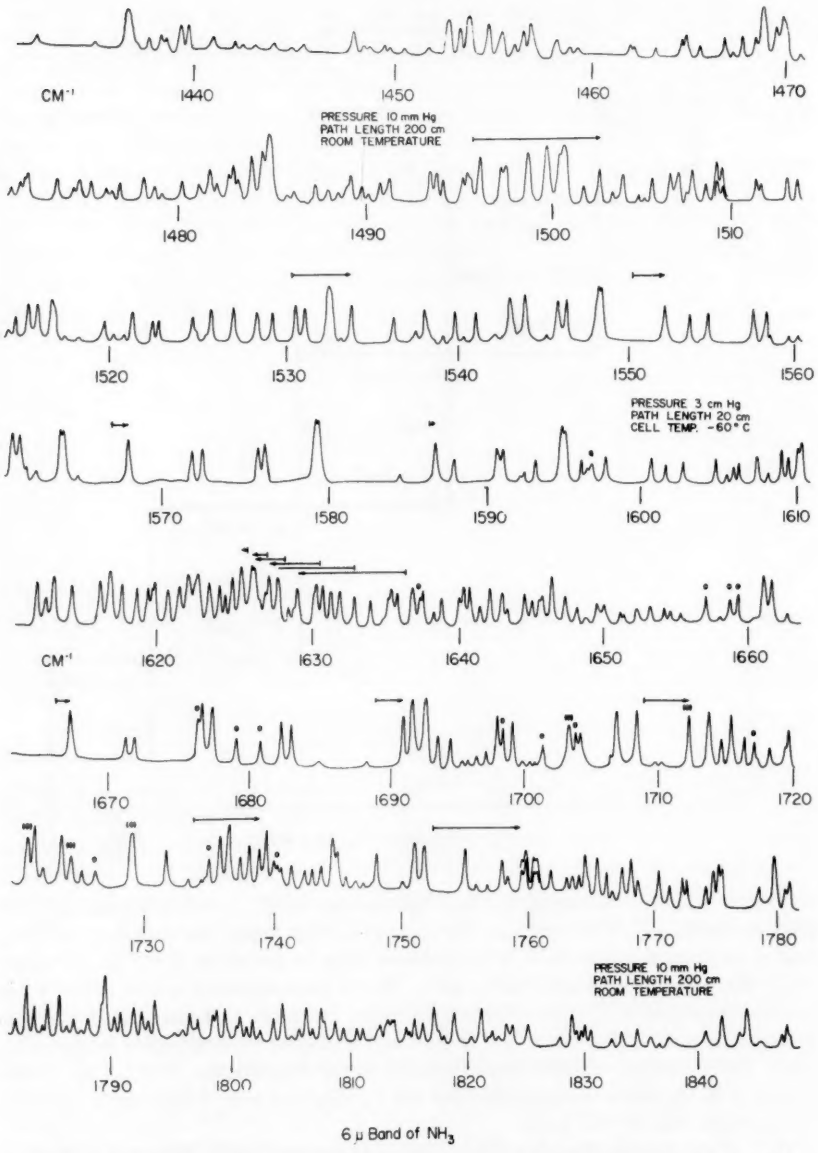

FIG. 2.—ν_2 in the infrared spectrum of NH_3. The arrows give the shift of the ${}^RP(J, 0)$, ${}^RQ(J, 0)$ and ${}^RR(J, 0)$ lines because of l-type doubling. The letters O and Q mark the R lines and Q branch, respectively, of the overtone band ν_2 $(2^a - 0^a)$.

detail by Coburn.[19] The grating was a 2,000-line/inch Bausch and Lomb replica grating used in second order. The gas was dried by passing it through $CaSO_4$ and then was placed in a 20-cm.-long cell which was cooled to $-60°$ C. to freeze out any residual water vapor. The extremities of the band were obtained at room temperature, using a path length of 200 cm.

Figure 2 shows the absorption pattern of the perpendicular band ν_4 studied first by Barker[20] and obtained later with more resolution by Wood, Bell, and Nielsen.[21] The increased resolution now obtainable makes it evident that most of the lines are split because of inversion doubling. This inversion doubling in a perpendicular band was first observed by Cummings[22] in the ν_3 band of NH_3 and has been studied by Benedict and Plyler[23] in the ν_3 band, as well as various combination bands involving the perpendicular vibrations. The statistics of the hydrogen nuclei in NH_3 permit only the antisymmetrical vibrational levels for J even and the symmetrical levels for J odd to exist when $K = 0$ in the ground state. Consequently, transitions originating from these ground-state levels give rise to lines in the spectrum which are single.

These transitions, i.e., the ${}^RP(J, 0)$, ${}^RQ(J, 0)$, and ${}^RR(J, 0)$ lines go to the upper levels for which $K = l = \pm 1$, which, as discussed in Section II, are split by the l-type doubling. The magnitude of the shift of the levels above and below the unperturbed position and consequently the corresponding shift in these lines is found experimentally to be given by $0.169\ J(J + 1)\ cm^{-1}$. Expanding the square root in equation (3) gives the total splitting of the levels to a good approximation as

$$\Delta\nu \doteq 4\ \frac{[\zeta_{2,4}{}^{(x)}]^2[B_e{}^{(xz)}]^2}{\delta}\ J(J + 1). \tag{4}$$

Comparison of the two results gives $\zeta_{2,4}{}^{(x)} = 0.754$. The shift of the lines corresponding to transitions from the lower level with $K'' = 0$ to the upper level with $K' = l = \pm 1$ and $J' = 0$–6 is illustrated in Figure 2 by the arrows where the tail of the arrow shows the position which the line would have if the l-type doubling did not exist. It is to be noted that the P and R lines are shifted toward higher frequencies and the Q lines toward lower frequencies. The theory as given above does not include the effect of inversion doubling, and it is assumed here that the l-type doubling is the same in both the antisymmetrical and the symmetrical vibrational levels of the perpendicular band. As was found in PH_3, AsH_3, and SbH_3, the Coriolis coupling factor, ζ_4, for NH_3 is also a function of K.

The lines marked with the letter O in Figure 2 belong to the R branch of the overtone band ν_2 ($2^s \leftarrow 0^a$) and were verified by using the energy levels of the ν_2 (2^s) state found by using the lines of the 10 μ fundamental ν_2 and the 16 μ difference band ν_2 ($2^s \leftarrow 1^a$). [The lines marked (O) are overlapped by lines belonging to the ν_4 band.] The interesting point here is the anomalous intensity pattern of the overtone band, wherein the R branch is quite strong, while the Q branch (marked Q in Fig. 2) is barely perceptible and the P branch does not show up at all, at the pressure shown.

Further analysis of the 6 μ band as well as the 10 μ and 16 μ bands is being carried out and will be reported on later.

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*ACETATE AS A CALCIUM-SPARING FACTOR IN NITROGEN FIXATION BY AZOTOBACTER VINELANDII**

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Studies[1, 2, 3] on the function of calcium in nitrogen fixation by *Azotobacter vinelandii* O revealed that this ion is required for the synthesis of metaphosphate by whole cells. Of the ions tested, it is the most potent stimulator of metaphosphate formation from adenosine triphosphate by cell-free extracts of this organism. In the course of these studies, it was observed that the rate of oxygen uptake of calcium-deficient cells in Burk's medium was stimulated by the addition of this ion. This stimulation is relatively insensitive to inhibition by 0.001 *M* sodium azide as compared to the sensitivity of calcium-deficient control cells. Roberts and Rakestraw[4] have reported a reversal of the azide inhibition of oxygen uptake by *A. vinelandii* O by the addition of ammonium acetate. This observation and that of Bulen and Frear[5] concerning the reversal of phosphite inhibition of growth and oxygen uptake of *A. vinelandii* by ammonium acetate led us to test the effect of acetate ion on the calcium requirement of *A. vinelandii* for nitrogen fixation.

Previous studies[1] have shown that low calcium ion concentrations in Burk's medium result in an extension of the lag phase of growth, as well as a decrease in the total growth of *A. vinelandii* O when using N_2 but not when NH_4^+ is supplied. We have now found that the addition of acetate or ethyl alcohol to Burk's sucrose-

salts medium partially replaces this requirement of calcium when N_2 is the source of nitrogen.

Materials and Methods.—Cultures of *A. vinelandii* O were maintained on a modified Burk's sucrose-salts liquid medium[6] and studied in 500-ml. Erlenmeyer shake flasks containing 100 ml. of this medium. The flasks were incubated at 30° C. on a New Brunswick rotary shaker rotating at 400 rpm. Tests for purity of culture were made periodically with the Gram stain and nutrient peptone technique described by Burk and Burris.[7]

Analytical reagent-grade chemicals were used for the preparation of media. Magnesium sulfate was prepared free of calcium contamination by recrystallizing twice from ion-free water. Ion-free water was obtained and glassware cleaned by methods previously described.[2] Growth curves were determined in specially made shake flasks with glass side arms made to fit the Klett-Summerson colorimeter. All readings were taken at 600 mμ.

Test media were inoculated with a 2 per cent by volume of a 24-hour shake culture of *A. vinelandii* in Burk's medium whose calcium concentration (as $CaSO_4 \cdot 2H_2O$) is 0.6×10^{-3} *M*. At times the inoculum was brought up on a medium containing only 20 per cent of the normal calcium concentration (0.12×10^{-3} *M*). The final concentration of calcium in the deficient media is that carried over in the inoculum and is specifically indicated in the legends of the figures. The pH of the test media containing salts of organic acids was maintained at 7.0–7.2 by the addition of dilute HCl at hourly intervals.

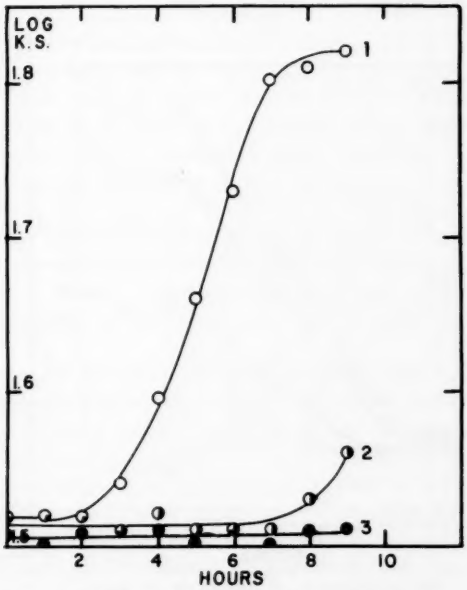

FIG. 1.—Effect of acetate on the growth of *Azotobacter vinelandii* O in a calcium-deficient medium. Burk's sucrose, N-free medium modified as follows: *1*—1.5×10^{-3} *M* phosphate; 1.2×10^{-5} *M* calcium sulfate; 1.5×10^{-2} *M* sodium acetate; *2*—6×10^{-3} *M* phosphate; 1.2×10^{-5} *M* calcium sulfate; *3*—1.5×10^{-3} *M* phosphate; 1.2×10^{-5} *M* calcium sulfate. The effect of a decrease in phosphate on the lag in a calcium-deficient medium is discussed by Esposito and Wilson, *Proc. Soc. Exptl. Biol. Med.*, **93**, 564–567, 1956.

Results.—The effect of acetate ion on the calcium requirement for nitrogen fixation is shown in Figure 1. Glucose, fructose, ribose, dihydroxyacetone, ethyl alcohol, sodium pyruvate, potassium glycolate, potassium glyoxylate, thioctic acid, and thiamine were all tested in the presence of sucrose; but, of these, only ethyl alcohol could be substituted for acetate. The failure of sodium pyruvate was not the result of the organism's inability to oxidize it, as the uptake and apparent oxida-

tion of pyruvate as measured by pH change of the medium was essentially at the same rate as acetate.

Most of the compounds tested are precursors of, or participants in reactions that form precursors of, acetate. The intermediates of the tricarboxylic acid cycle may be considered products of acetate metabolism, since the azotobacter contains a complete tricarboxylic acid cycle and means for the entry of acetate.[8] Recrystallized malic acid neutralized with potassium hydroxide when added to Burk's sucrose medium, however, did replace the need for added calcium ion. However, this replacement may be the result of a "jamming" of the metabolic pathways, with a

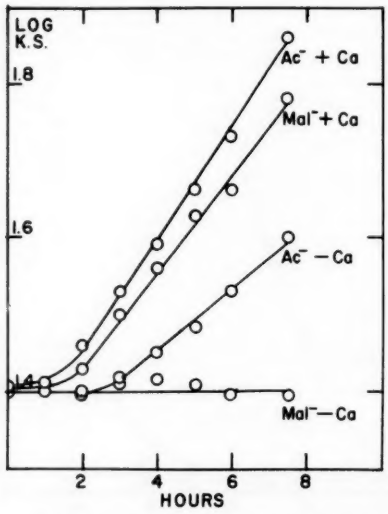

FIG. 2.—Growth of *Azotobacter vinelandii* O in Burk's medium with acetate and malate as sole carbon sources and high and low levels of calcium. *Ac⁻*: 1.5×10^{-2} *M* sodium acetate; Mal^-: 1.5×10^{-2} *M* sodium malate; *+ Ca:* 0.5×10^{-3} *M* calcium sulfate; *−Ca:* 2.5×10^{-6} *M* calcium sulfate.

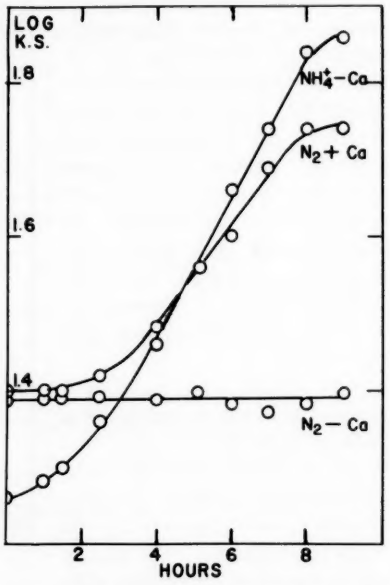

FIG. 3.—Comparison of effect of calcium on the growth of *Azotobacter vinelandii* O using free and combined nitrogen with malate as sole source of carbon. Medium was Burk's mineral salts plus 1.5×10^{-3} *M* potassium malate. NH_4^+: 8×10^{-3} *M* ammonium sulfate; *+Ca:* 0.6×10^{-3} *M* calcium sulfate; *−Ca:* 2.5×10^{-6} *M* calcium sulfate.

resultant spillover to acetate or a compound formed from it. This explanation is indicated by the results contained in Figure 2. When malate is added as the sole carbon source for the organism, adequate calcium is necessary for nitrogen fixation to be initiated. However, if acetate is added as the sole carbon source, nitrogen fixation is initiated in the calcium-deficient medium.

One possible explanation of the failure of malate added as the sole carbon source to replace calcium is a failure of sucrose-grown cells to adapt to the utilization of malate. This would necessarily result in an extension of the lag phase of growth and indicate an apparent lack of calcium-sparing activity. Figure 3 demonstrates that this is not so. The addition of either calcium or ammonium ions to a medium

of Burk's mineral salts plus potassium malate results in a normal growth curve. This indicates that the cells adapt to malate within a normal period of lag and that the utilization of malate in a calcium-deficient medium is adequate for the assimilation of ammonium ion but not for molecular nitrogen.

An examination of Figure 2 reveals that, although the presence of acetate in the calcium-deficient medium suffices for the *initiation* of growth, the addition of calcium results in a *higher* rate of growth. If, however, sucrose is present in addition to acetate, the rate of growth is as high as that of cells in the presence of acetate and adequate calcium. Preliminary results suggest that acetate plus malate also results in a high rate of growth in the calcium-deficient medium.

Discussion.—Studies[9, 10, 11] on the kinetics of incorporation of molecular nitrogen into the organic pool of the cell indicate that ammonia is the major terminal inorganic form of the newly fixed ("juvenile") nitrogen prior to formation of amino acids. If so, obviously, the tricarboxylic acid cycle could play an important role as a source of acceptors for the ammonia—e.g., α-ketoglutaric acid. The data reported here, however, suggest that some other compound(s) apart from those of the tricarboxylic acid cycle may function in the trapping of the "juvenile" nitrogen or have some other role in the formation of ammonia from molecular nitrogen. When ammonium ion is supplied, malate functions satisfactorily as a source of carbon in the presence of limited calcium, ($2.6 \times 10^{-6} M$) but, if molecular nitrogen is the nitrogen source, adequate calcium, acetate, or sucrose must be available for the initiation of growth.

It seems, then, that calcium functions in an indirect manner in nitrogen fixation by *A. vinelandii* O, apparently being necessary for the formation of acetate or of a compound related to acetate that is required for the fixation reaction. Whether acetate is necessary for the formation of an acceptor of "juvenile" nitrogen at a level preceding ammonia or serves some other function is as yet unknown. One possibility that has been tested was directed toward providing evidence that hydroxylamine is a precursor of ammonia. If so, it could combine with acetyl-CoA to form acetohydroxamic acid; attempts to demonstrate utilization of this compound by whole cells and by cell-free extracts were unsuccessful.

Summary.—The requirement of calcium ion for nitrogen fixation by *A. vinelandii* O is relieved when sodium acetate is supplied the organism. For example, at a calcium ion level of 10^{-5}–10^{-6} M the organism will not initiate growth on N_2 but will on NH_4^+. Addition of sodium acetate to the calcium-deficient medium allows growth to occur in the absence of fixed nitrogen. Ethyl alcohol alone or potassium malate plus sucrose also can replace this calcium requirement. The following compounds could not substitute for acetate in this calcium-alleviating role: glucose, fructose, ribose, dihydroxyacetone, sodium pyruvate, potassium glycolate, potassium glyoxylate, thiamine, or thioctic acid.

* Supported in part by grants from the Rockefeller Foundation and the Research Committee of the Graduate School from funds provided by the Wisconsin Alumni Research Foundation.

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*CELLULAR PROLIFERATION IN THE MOUSE AS REVEALED BY AUTORADIOGRAPHY WITH TRITIATED THYMIDINE**

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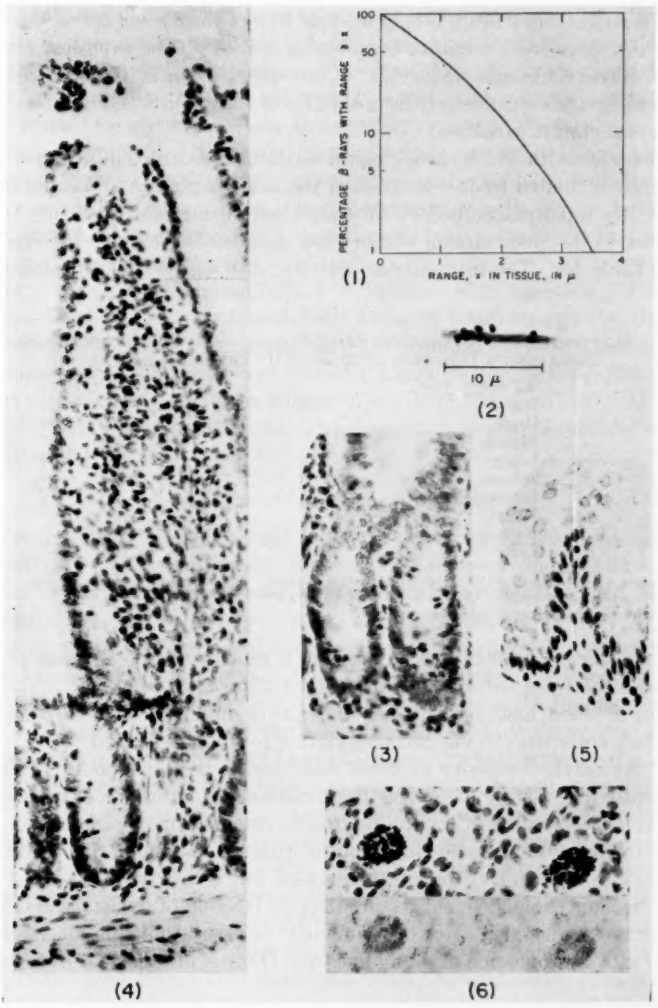
Communicated by Donald D. Van Slyke

INTRODUCTION

Many cell populations within the body undergo constant breakdown and renewal.[1] Classical histological techniques do not allow adequate study of dynamic processes, and autoradiography is proving increasingly valuable for following these processes at the cellular level. In order to distinguish cell renewal from the renewal of cellular constituents, the label must be incorporated into a fixed component of the cell which is not lost during the cell's lifetime; and present knowledge suggests that a label incorporated into deoxyribonucleic acid (DNA) should be most useful for this purpose.

DNA occurs within the nucleus of the cell associated with the chromosomes and appears, in fact, to be the bearer of the genetic information which each cell passes on to its descendants. Since this information must be handed down through countless generations, extreme immutability of the DNA molecules would seem desirable, and, in fact, evidence is accumulating that DNA in a cell is never replaced. This evidence, as reviewed[2] by Thorell and Smellie, shows that DNA is metabolically inert in resting cells. The possibility of turnover during cellular division, which these authors considered, would appear to be denied by more recent reports. Thus Taylor, Woods, and Hughes[3] used tritiated thymidine (thymine occurs uniquely in DNA, and thymidine is incorporated efficiently and exclusively into DNA[4]) to label the new DNA of bean roots. Autoradiography showed that chromosomes are transmitted to daughter cells as *intact half-chromosomal units.* This suggests that duplication of the genetic material involves the separation of each chromosome into complementary halves and the formation of new complements upon the existing halves as templates. *Escherichia coli* labeled with tritiated thymidine distributed their DNA among their progeny in a like fashion.[5] In both these cases no loss of label from the total population was observed over several cell generations.

Autoradiographic identification of cells should permit a more detailed analysis of cellular dynamics. The specific advantage[6] of tritium for autoradiography lies in the very high resolution which can be obtained because of the very weak energy and consequently short range of its β radiation (Fig. 1). The maximum range in



FIGS. 1–6.—Fig. 1, range-frequency curve for β-rays of tritium (based on data of Robertson and Hughes). Fig. 2, chain of *E. coli* (thymine-requiring mutant) made up of 2 labeled and 1 or 2 unlabeled cells. Note resolution of approximately 1 μ. Fig. 3, jejunum of mouse 7 hours after injection of thymidine (Harris hematoxylin). 175×. Label in crypts only. Fig. 4, jejunum of mouse 48 hours after injection of thymidine (Harris hematoxylin). Label diluted in crypts and distributed along entire villus. Fig. 5, forestomach of mouse 7 hours after injection of thymidine. (Feulgen) Label in basal-cell layer. Fig. 6, polymorphonuclear leukocytes in intestine, 63 hours after injection of thymidine, 48 hours after irradiation (2,000 rad, whole body). (Feulgen) Photographs with and without filter. *Upper photograph*, tissue; *lower photograph*, grains.

tissue of a β-ray from tritium is only 6 μ and half of the β's will travel less than 1 μ. The net distances from origin to terminus of tracks will be even less because the direction of travel changes frequently. Consequently, the activated silver grains of an autoradiogram should largely lie within 1 μ of their source. Figure 2 shows how well this expectation is realized.

The precision with which chronological relationships can be established via autoradiography is limited by the duration of the labeling phase. Tritiated thymidine is apparently rapidly absorbed by all cells when injected intravenously into mice, since it leaves the blood stream within a few minutes and may then be found in all tissues (Table 1). The intracellular activity, although soluble in alcohol, is not

TABLE 1

DISTRIBUTION OF ALCOHOL-SOLUBLE* RADIOACTIVITY 15 MINUTES AFTER INTRAVENOUS INJECTION OF 20 μC OF H^3 THYMIDINE INTO MOUSE

Tissue	Counts† per Mg
Blood	70
Brain	65
Muscle	60
Lung	60
Spleen	70
Intestine	75
Heart	80
Kidney	130
Liver	260

* The tissues were homogenized in 50 parts of absolute alcohol. Ten per cent TCA extracted similar, but somewhat larger, amounts of activity. (About 30 c/mg of activity was volatile, presumably water.)

† At about 5 per cent counting efficiency.

thymidine, since, in contrast to thymidine, it remains in the aqueous phase upon distribution between saturated ammonium sulfate and *n*-butanol. The activity remaining in blood, however, still distributes as thymidine. This suggests that thymidine may enter the cell via phosphorylation to thymidylic acid.

The "thymidylic" activity of tissue falls during the next hour. This may be conveniently followed by measuring the simultaneous formation of tritium hydroxide. The concentration of tritium hydroxide reaches a maximum within about 45 minutes following intravenous injection or within 1 hour following intraperitoneal injection. Consequently, the precursor pool for DNA synthesis must simultaneously become exhausted, suggesting that all labeling of nuclei occurs during the first hour after injection. However, it should be noted that this conclusion implies a similar rate of exhaustion of thymidine from DNA-synthesizing cells as from tissue as a whole.[7]

The following observations are based on autoradiographic examination of twelve mice, no two of which received identical treatment. The results, though obviously preliminary, seem sufficiently exciting to warrant this communication.

MATERIALS AND METHODS

Tritiated thymidine of a specific activity of approximately 300 curies/mole was obtained from the Schwarz Laboratories. It was labeled in the pyrimidine portion of the molecule (presumably by exchange with that hydrogen bound to carbon in the pyrimidine ring), and it was free of labeled impurities as judged by recrystallization with carrier thymidine. It was diluted with isotonic sodium chloride to a convenient concentration for injection.

Young adult male C57 brown mice were injected intraperitoneally with 0.7 μc of tritiated thymidine per gram mouse. Animals were sacrificed at intervals, having been fasted for 12 hours previously. (This brief fasting period yields intestines which are nearly empty and does not seem to affect cell production in the gastrointestinal tract; we are not certain about other organs.) Tissues were fixed in neutral formalin, imbedded in paraffin, and sectioned. Autoradiograms were made with stripping film as described by Pelc[8] and developed after 1–2 months' exposure. Staining was with Feulgen before autoradiography or with Harris hematoxylin after photographic development. Autoradiograms so prepared show essentially no grains except over labeled nuclei.

Six normal mice were sacrificed from 7 to 48 hours after injection. Three mice were X-irradiated 7 hours after labeling and three, 18 hours afterwards; these mice were sacrificed 7–47 hours after irradiation. X-irradiations were administered to the whole body with 250-kvp X-rays filtered through $^1/_4$ mm. of Cu + 1 mm. of Al, at a target distance of 50 cm., with a mean dose rate of 156 rads/min. An average dose of 2,000 rads was administered. This dose leads to acute intestinal radiation death in all mice.

OBSERVATIONS ON NORMAL ANIMALS

Gastrointestinal epithelia.—The epithelial lining of the various portions of the gastrointestinal tract is continually renewed. The zones where cells originate, spend their functional life, and finally end by being desquamated into the lumen occupy separate regions, which are, by and large, well known.

Seven hours after intraperitoneal injection of the thymidine solution, labeled cells were found in the generative zones: the basal layer of the forestomach (which is lined with stratified epithelium) (Fig. 5), the gland necks of the fundus, the crypts of the pyloric region (particularly the lower third, except for the cells at the very bottom of the crypts), the crypts of the small bowel (including the region occupied by the Paneth cells; however, no label was seen in cells containing granules) (Fig. 3), the crypts of the colon. The fraction of labeled cells ranged from about one-fifth in the forestomach, through one-third to one-half in stomach and colon, to about two-thirds in the jejunum. The amount of label per cell appeared to be fairly uniform. Most mitoses seen at this time were strongly labeled (in a subsequent series, labeled telophases were found as early as $2^1/_2$ hours after injection).

In preparations taken 14–48 hours after injection, labeled cells were seen to have moved from the generative to the functional zones; concomitantly, the number of labeled cells in the generative regions decreased, as did the amount of label per cell (Fig. 4). An exception seems to be the colon, where a moderate number of heavily labeled cells remained near the bottom of the crypts after 48 hours. The movement of labeled cells was not very regular. This was best seen in the jejunum, with its large percentage of labeled cells: the zone behind the leading edge was characterized by patchy labeling, and the distance to which the label progressed varied considerably among villi and even on the two sides of a single villus. A substantial fraction of the cells appears to pass through a "pool" which is composed of the intestinal lining between the mouths of the crypts and the bases of the villi. Cells passing through this pool migrated up the villus in a wave, which seems to be about 15 hours behind the leading edge.

In fundus, pylorus, jejunum, and colon the distance from generative zone to surface was covered in less than 2 days, as shown by the appearance in the lumen of desquamated labeled cells 48 hours after injection (Fig. 4). In the same 48 hours, in the forestomach, the label moved into the prickle-cell layer but did not reach the surface. In the depth of the fundus glands, a few labeled cells were found at this time but no fully differentiated chief and parietal cells. In the forestomach and fundus, the life-span of the adult cells is seen to be more than 2 days.

The percentage of labeled mitoses was nearly 100 in the preparation taken 7 hours after injection; it was very low 14 hours after injection and showed irregular variations (between 20 and 60 per cent) in subsequent samples.

Spleen.—The most heavily and frequently labeled cell was the normoblast; the label seemed to stay constant for some time but appeared reduced at 48 hours. Labeled megakaryocytes were found in all preparations, the percentage labeled possibly increasing with time up to about 60 per cent. In the Malpighian follicles, a few reticulum cells and about 10 per cent of the lymphocytes were labeled; there were some groups of labeled cells in the germinal centers. The amount of label in lymphocytes was smaller than in the generative cells of the gastrointestinal tract. During the first day after injection, the percentage of labeled lymphocytes increased and had decreased at 48 hours. A few doughnut-shaped myelocytes were labeled in all specimens; at 48 hours, there were large numbers of labeled mature granulocytes.

Other Tissues.—In the first series of animals, the pancreas contained label only in some duct cells; in a later series (not fasted) several well-labeled cells and mitoses were found. Occasionally, fibroblasts, and, rarely, smooth-muscle cells were labeled in all preparations. A labeled liver cell was infrequently found. An occasional labeled cell was found in Brunner's glands.

OBSERVATIONS ON X-IRRADIATED ANIMALS

In the intestine, labeled cells contributing to the intestinal debris were seen in all irradiated animals. Nuclear debris in lymph follicles and germinal centers was unlabeled in animals X-irradiated 7 hours after injection and were labeled in one animal X-irradiated 18 hours after injection. In one animal sacrificed 2 days after X-irradiation, there was a striking number of labeled macrophages (Fig. 6).

In the gastrointestinal tract the migration of the labeled cells from the generative zone to the surface was not markedly affected by irradiation with 2,000 rads. Also, the maturation and function of the leukocytes did not seem to be affected, since many labeled leukocytes were found inside the glands of the stomach and intestines at 48 hours (Fig. 6).

Discussion.—These results indicate that tritiated thymidine is a powerful tool for studying physiological processes involving the proliferation, maturation, and migration of cells in normal and pathological states.

Nuclei labeled with tritiated thymidine appear to be marked permanently, subject only to dilution of the label by cell division. Factors that might cause underestimation of the number of labeled cells are (1) dilution of the label by so many cell divisions that the label would escape detection (under the conditions of the present experiments this might occur after three or four divisions); (2) loss of tritium by exchange with water. Such loss appears improbable because the tritium in the labeled thymidine is stable in water even in the presence of acid or alkali.

The quantitative estimation of radioactivity in each nucleus by grain counts would be useful in determining the number of cell divisions subsequent to labeling, since each division should halve the radioactivity per nucleus. However, the technical difficulties are considerable. The very short range of β-rays from tritium limits the average number of silver grains activated per disintegration to approximately 1 with tritium dissolved in the emulsion. In actual practice, geometrical factors sharply lower autoradiographic efficiency; we estimate that there is about 1 grain per 5 disintegrations for labeled bacteria and about 1 grain per 20 disintegrations for labeled nuclei in smears of mammalian cells. The low yields of grains can be compensated, to a degree, by long exposure times because the half-life of tritium is long (about 12 years). In tissue sections the situation is further complicated by the fact that the thickness of the section is several times the range of most of the β-rays. Therefore, labeled loci not very near the cut surface may not register at all. The number of grains showing above a labeled cell will depend on both the amount of label in the cell and the distance between it and the emulsion; this distance, in turn, depends not only on interposed tissue but can also be significantly altered by unavoidable imperfections in apposition of stripping film to tissue. Accordingly, estimates of the amount of label in cells in section can be made only in comparison with other cells situated in the same plane and are never precise. More quantitative studies by grain count may be possible by the study of imprints and smears where the geometry is more favorable.

One has also to consider the possibility that labeled cells may not behave like normal ones. In the experiments reported here, the grain count over maximally labeled cells (about 20 grains after 2 months' exposure) indicated that they contain of the order of 10^5 atoms of tritium per cell. This calculation is based on the fact that tritium disintegrates at the rate of 0.5 per cent per month and assumes that there are about 50 disintegrations per grain formed. The labeled thymine was therefore a small fraction of the total thymines (about 10^{10}) contained in a nucleus.

We can further estimate that the amount of thymidine (labeled and unlabeled) added in our experiments must have been diluted one thousand fold in the cell during its conversion into new DNA. Hence it appears that the added thymidine could not significantly affect the size of the thymidine pool or the kinetics of thymidine incorporation into DNA.

On the other hand, one cannot exclude the possibility that radiation from tritium has altered cellular physiology. A cell labeled as in our experiment experiences about 1 disintegration per hour, and the average 5.7 kev of energy liberated (the equivalent of 200 ion pairs) is largely absorbed within the nucleus. We have not noticed any marked effect of the label upon subsequent cell divisions or cell survival; but our study has been limited to periods up to 2 days and to a few kinds of cells. In tissue culture of HeLa cells, labeling levels at least one hundred times higher appear necessary to inhibit cell proliferation.[9]

In cell populations which are homogeneous with respect to cell type and are synchronized, the evaluation of labeling studies is straightforward. In mammalian tissues these conditions are not fulfilled but can be approximated by producing certain conditions, such as regeneration, or by considering only cells at a particular well-defined stage, such as mitosis. All cells labeled by a single brief exposure to tritiated thymidine must have been synchronized in the sense that they were forming DNA at the time of labeling.

If a cell population is homogeneous and completely asynchronous, then the percentage of labeled cells present after a brief labeling period measures the percentage of the total generation time during which cells can take up thymidine and use it for DNA synthesis. If the generating zones of the gut are thus considered as homogeneous populations of cells, it follows from our results that the synthetic time must vary from 20 to 70 per cent of the generation time in different regions. (In tissue cultures of bone marrow[10] or HeLa cells[11] the synthetic time is approximately 30 per cent of the generation time.) If the durations of the periods of synthesis in these different regions are similar, then the rate of cell renewal must vary directly with the fraction of labeled cells. In agreement with this, labeled cells moved more rapidly out of the heavily labeled crypts of the jejunum than out of the more sparsely labeled basal epithelium of the forestomach.

With regard to intensity of labeling, throughout the gastrointestinal tract those cells that were labeled at all appeared to be labeled with equal intensity, also suggesting similar synthetic rates. By way of contrast, lymphocytes were weakly labeled. This may be interpreted as possibly indicating a slower synthetic rate for the lymphocyte. However, in addition to the inaccuracies resulting from variation in autoradiographic efficiency, variation in cellular uptake of the isotope or in size of the precursor pool might explain this difference.

The time required for the appearance of labeled mitotic figures following injection measures the interval between completion of DNA synthesis and the beginning of mitosis. Since all mitoses were labeled 7 hours after injection (the shortest time studied), all cells must proceed into mitosis within 7 hours following the completion of DNA synthesis. In the subsequent intervals studied, the percentage of labeled mitoses decreased to a low value at 14 hours and then varied between 20 and 60 per cent. It is possible that further studies over more frequent time intervals may show a rhythmic variation in labeling, which should correspond to the generation time.[10]

Radiation injury affects the gastrointestinal tract by stopping the renewal of its epithelia. With a sufficiently high dose, the generative compartments are largely emptied, and the production of new cells is blocked. Death may ensue if the functional compartment is depleted before regeneration can provide replacement. It has been postulated that the generative compartment is largely emptied by *cell death* and that the functional compartment remains populated, for a time, with those cells which were in it at the time of irradiation.[12] This picture must be modified on the basis of the present studies: the generative compartment is emptied not only by cell death but also by cell migration, since cells continue to migrate into the functional compartment after 2,000 rads of X-irradiation.

Summary.—Tritium carried in thymidine has been used to label cell nuclei of mouse tissues by incorporation into deoxyribonucleic acid. Autoradiograms show surprisingly large numbers of labeled nuclei, suggesting that the amount of cellular proliferation is greater than has been inferred from the study of mitotic indices. The period for deoxyribonucleic acid synthesis appears to be a large fraction of the generation period in the intestinal mucosa. Cellular migration in the gastrointestinal epithelium from the crypts to the tips of the villi is clearly demonstrated by this technique, and variations in the rate of migration are seen in different regions. Two thousand rads of X-rays selectively inhibit cell production without markedly

affecting the maturation or migration of cells into functional compartments. The possibilities and limitations of tritiated thymidine for further studies are discussed.

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*DIFFERENCE IN ELECTRIC POTENTIAL ACROSS THE PLACENTA OF GOATS**

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The data presented in this report were obtained during part of an investigation[1] directed primarily to study the "forces" that determine the net transfer of water across the placenta from the maternal to the fetal blood and the mechanisms that regulate the water content of the fetus and the surrounding cavities. To learn whether electro-osmotic phenomena operate in the process of transfer of water across the placenta, we have proceeded to determine whether or not any difference in electric potential exists across the barrier that separates the maternal from the fetal blood.

The animals used for the experiments were goats (duration of pregnancy 145–47 days), bred on known dates. The heparinized mother was under spinal anesthesia (Pontocain) and received, in addition, a light dose of thiopental sodium (Pentothal)

intravenously. Polyethylene tubes, filled with an isotonic solution of NaCl, were introduced into a maternal femoral artery and a maternal jugular vein and in one, sometimes two, small branches of the umbilical vessels. A polyethylene tube was introduced at the same time into the allantoic cavity by passing it through the uterine wall. The difference in potential between any pair of such catheters was measured by dipping their free ends into two beakers filled with isotonic NaCl in which the tips of two calomel electrodes were immersed. The calomel electrodes were connected to a Beckman pH meter, Model G. As was to be expected, no appreciable difference in potential could be measured between the femoral artery and the jugular vein of the mother. Similarly, on the fetal side, no difference in potential was detectable between the fetal vessels. In all cases a considerable difference in potential existed between the maternal and the fetal blood streams, and in every case the fetus was negative with respect to the mother (see Table 1).

TABLE 1

DIFFERENCE IN POTENTIAL BETWEEN MATERNAL AND FETAL BLOODS

Fetal Age (Days)	Millivolts
79	+133
85	+ 90
90	+100
96	+100
105	+115
110	+ 55
110	+ 70
111	+ 85
119	+ 50
131	+ 25

If the data are tabulated according to fetal age, as in Table 1, it becomes apparent that, under the experimental conditions in which we measured it, the difference in potential was greater in the younger fetuses. In some experiments a small incision was made through the uterine muscle, to expose the outer face of the chorion, and the tip of a polyethylene tube filled with isotonic NaCl solution was pressed against the surface of the chorionic membrane. The difference in potential between this catheter and a catheter placed beneath the chorion, either in the allantois or in a fetal vessel, was of the same sign and magnitude as that measured between the fetal and maternal bloods. Thus the chorionic membrane appears to be the source of the electromotive force that creates the difference in potential across the placenta.

At present we are inclined to think that the recorded difference in potential is the electric manifestation of an active transfer of ions across the placenta. This hypothesis is based upon an analogy with other biological systems that have been more thoroughly investigated, as, for example, the skin and the gastric mucosa of the frog.[2] In addition, the available experimental evidence tends to indicate that some ions—for example, the amino acids of the maternal plasma[3]—are actively transferred from the mother to the fetus.

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† Public Health Service Research Fellow of the National Heart Institute.

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ERRATA: The Formation of Antibodies in Man after Injection of Pneumococcal Polysaccharides by Michael Heidelberger.

In the article of the foregoing title appearing in these PROCEEDINGS, **43,** p. 884, Table 1 should appear as follows:

TABLE 1

MICROGRAMS OF TYPE-SPECIFIC ANTIBODY NITROGEN PER 4 ML. OF SERUM FROM ALL SUBJECTS ALL TYPES INJECTED, 3 WEEKS TO $5^1/_2$ MONTHS AFTER INJECTION OF 1–6 POLYSACCHARIDES

μG. ANTIBODY N/4 ML SERUM	S I		S II		S III		S V		S VII		S VII	
	113 *	%	80	%	33	%	96	%	48	%	34	%
0–10	31	27	16	20	12	36	69	72	7	15	17	50
11–20	31	27	19	24	7	21	13	14	9	19	8	24
21–30	18	16	17	21	8	24	5	5	6	13	4	12
31–40	10	9	11	14	0	0	5	5	7	15	1	3
41–50	5	4	4	5	2	6	1	1	1	6	2	6
51–60	5	4	0	0	1	3	1	1	3	6	1	3
61–70	1	1	6	7	0	0	0	0	5	10	0	0
71–80	2	2	1	1	1	3	0	0	2	4	0	0
81–90	1	1	3	4	0	0	0	0	0	0	0	0
91–100	1	1	0	0	2	6	0	0	1	2	1	3
101–200	6	5	2	3	..	..	2	2	5	10	..	..
>200	2	2	1	1	..	..	..	..	..	..	..	..

* No. of subjects.

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